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EFFECTS OF MALATHION ON WATER CONTENT
AND METABOLISM IN BLATTELLA GERMANICA L.

by

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ABSTRACT

The effects of malathion on water loss, free amino acid concentrations of the blood, and metabolism of glucose $(U)C^{14}$, glutamate $(U)C^{14}$ and glycine $(U)C^{14}$, in susceptible and resistant strains of Blattella germanica Linnaeus were studied.

There is a positive correlation between depletion of blood volume, and the degree of malathion intoxication.

The increased metabolic activity of the malathion-treated susceptible strain of roaches resulted in a large depletion of free glycine, proline, glutamate, and glutamine of the blood. Malathion had no effect on the glutamine of the resistant roaches though glutamate, glycine, and proline were slightly reduced. This indicates a selective correlation between the degree of intoxication and the depletion of amino acid reserves. It is suggested that most of the depletion of free amino acids was caused by their utilization as a readily available substrate for the Krebs cycle for energy production.

There was an increase in catabolism of injected labelled compounds in the malathion-treated roaches of the susceptible strain, which resulted in a greater production of $C^{14}O_2$ than in the controls. The incorporation of the labelled carbons of glucose $(U)C^{14}$, into carbohydrates, proteins and amino acids; that of glutamate $(U)C^{14}$ into carbohydrates and other amino acids and that of glycine $(U)C^{14}$ into amino acids was less in the intoxicated susceptible roaches than the controls. The rate of catabolism of injected labelled compounds in the malathion-treated resistant strain was

about the same as that of the susceptible untreated controls.

The depletion of free hemolymph glutamine in the malathion-treated roaches indicates that a complete breakdown of one of the mechanisms of ammonia disposal had occurred. And an increase in the total nitrogen, non-protein nitrogen and urea of the blood was observed. Therefore, ammonia toxicity may be a contributing factor in the mode of action of malathion.

There was, initially, a slight inhibition of cholinesterase in the malathion-treated resistant strain but normal activity was eventually restored. The initially depleted free glutamate and proline also showed similar restoration. These observations suggest the presence of a detoxifying mechanism with an accelerating effect of detoxification.

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DEFINITION OF TERMS

Aliesterases	: Enzymes which hydrolyse short-chain aliphatic carboxyesters but have no action on acetylcholine.
Cholinesterases	: Enzymes hydrolysing acetylcholine.
DDT	: 2, 2-bis-(p-chlorophenyl) -1, 1, 1-trichloroethane.
DFP	: Di-isopropyl fluorophosphate.
KD50	: Time at which 50 per cent of the malathion treated population is knocked down.
LC50	: Concentration of malathion ($\mu\text{l/sq. cm.}$) required to kill 50 per cent of the treated population in a given time.
Lethal dose	: Concentration of malathion ($\mu\text{l/sq. cm.}$) required to kill the whole treated population in a given time.
Trehalose	: (1-(α - D - glucopyranosyl) α - D glucopyranose.

I. INTRODUCTION

The mode of action of insecticides and the mechanism of resistance to these chemicals in insects of both medical and agricultural importance has occupied a most important position in toxicological research. The term 'mode of action' is a broad one and involves the study of the sequence of biochemical reactions started or effected by toxicity and leading to the death of the insects. Some of these reactions cause physiological lesions which may be of primary importance and which may lead to secondary lesions or to various other abnormal but unimportant reactions. Thus a final lesion leading to death may be a direct or indirect result of some primary lesion.

The chain of events in organophosphate poisoned mammals that leads to death, is fairly well accepted. The mode of action starts with the inhibition of cholinesterase (primary effect), accumulation of acetylcholine (secondary effect) which disrupts the nervous system causing the failure of the respiratory apparatus. Death is then brought about by asphyxiation. Thus the inhibition of cholinesterase and failure of respiratory centres are of great mortal importance.

In insects, knowledge of the mode of action of organophosphates is limited. Experiments conducted so far have demonstrated the existence of the mammalian-like primary and secondary effects, but little is known about the ultimate cause of death in insect.

Thus far, the strongest evidence of a mammalian-like primary lesion in insects has been afforded by the correlation between the accumulation of acetylcholine (Smallman and Fisher 1958) due to inhibition

of cholinesterase (Mengle and Casida 1958) and the death of insects. On the other hand, recovery of cholinesterase activity in intoxicated and dying insects (O'Brien 1956) and the non-toxic action of acetylcholine have also been reported (Hopf 1954). The hypothesis of cholinesterase inhibition was further weakened by Van Asperen (1958) who reported far greater inhibition of aliesterase than cholinesterase by four different organophosphates. However, aliesterase inhibition is not thought to be important as the enzyme complex is present in low concentration in resistant strains of insects (Van Asperen and Oppenoorth 1959). Triorthocresyl phosphate has also been reported to inhibit aliesterase to a great extent in houseflies, without affecting the cholinesterase or inducing any toxic symptoms (Stegwee 1959).

Hydrolysis of various other esterases was also observed to be inhibited by organophosphates (O'Brien 1960).

An elucidation of the mechanism of resistance to insecticides would certainly unravel to a great extent, the mode of action of insecticides. Attempts have been made to correlate resistance with differences in the rate of penetration of insecticide through the body (Krueger et al. 1960), rate of degradation of insecticides by insects (Mengle and Casida 1960) and the differences in the amount of esterases (phosphatases) in the insects of susceptible and resistant strains (Van Asperen and Oppenoorth 1959). Mengle and Casida (1960) have demonstrated that methyl parathion inhibited much less cholinesterase in resistant roaches than in susceptible strain. Recently Ray (1963) has shown with gas chromatography that nerves of resistant and susceptible American cockroaches, treated with equal amounts of dieldrin contained the same amount of undegraded insecticide.

From the existing evidence, it is clear that of all the systems studied in the resistant and susceptible strains of insects, the inhibition of cholinesterase by organophosphates is probably the most significant primary lesion. The final lesions that lead to death are likely to be found by carefully studying the consequences of the secondary lesions, such as, the intermediary metabolism of carbohydrates and proteins, utilization of high energy bonds and the blood-borne hormones.

Sternburg and Kearns (1952, 1959) have demonstrated the presence of toxic neurohormones in the blood of DDT poisoned American roaches. The direct or indirect action of organophosphates on nerve functions could upset the hormonal balance and thus affect a number of physiological and biochemical activities in insects. Some of the neurotoxins, which were reported to be amines, indicate the possibility of a close relationship between protein metabolism, accumulation of nitrogenous metabolites and neurotoxins.

Ludwig (1946) has suggested the exhaustion of carbohydrate reserves in DDT-treated Japanese beetle larvae, as the cause of death. However, administration of glucose failed to save DDT poisoned roaches (Merrill et al. 1946). Symptoms similar to starvation, such as an increase in blood amino nitrogen, total nitrogen, and urea have been reported in DDT poisoned insects (Ludwig and Bartolotta 1953, Joseph 1958). Winteringham and Harrison (1956) observed the depletion of free proline in DFP treated houseflies, as did Corrigan and Kearns (1963) in DDT poisoned roaches. These observations suggest the possibility of ammonia toxicity to insecticidally intoxicated insects.

The present study was undertaken to determine the effect of malathion toxicity on the metabolism of free amino acids of the blood of Blattella germanica L. and on amino acid carbohydrate interconversion. The scope of the study has been extended to a malathion resistant strain of the German roach (appendix, page 109) in order to study the relationship between the action of malathion on the free amino acids and the development of resistance by the roaches to this insecticide. The present studies are expected to add to the knowledge of factors contributing to malathion toxicity to insects, including the role of primary and secondary lesions in bringing about the physiological lesion which causes death in intoxicated individuals.

II. TOXICOLOGICAL STUDIES

1. Introduction:

An insecticide interferes with various biochemical and physiological reactions in the insect body. Some of the physiological lesions caused by insecticides are of secondary nature, such as loss in weight and depletion of carbohydrate reserves, high energy phosphates, free amino acids of the blood and the water content of the blood and body. Various workers cited below have studied water and weight loss in poisoned insects and have explained it on the basis of increased regurgitation, defecation, transpiration and redistribution of available water within the body.

Buck et al. (1952) reported about ten to twenty per cent loss in weight of DDT-poisoned Phormia regina (Meigen), due to increased regurgitation. Ludwig (1946) reported greater elimination of fecal matter by hyperactive DDT-intoxicated Popillia japonica (Newman). Jochum (1956) observed general defecation and discharge of large quantities of orange coloured liquid from the mouth of parathion-treated Leptinotarsa decemlineata (Say), and attributed the majority of weight and water losses to increased regurgitation. Similarly, silk-worm larvae, treated with an organophosphate were observed to lose about 32 per cent of their wet weight while the poisoned but ligatured insects did not suffer any loss. Increased regurgitation should be expected if the insecticides were to stimulate contraction of the gut by affecting the nervous system. Movement of the fore gut of the desert locust has been demonstrated by Clarke and Grenville (1960) to be under the control of the ingluvial ganglion. However, Jochum (1956) had observed greater activity of the salivary glands in parathion-intoxicated silkworms.

Buck and Keister (1949) reported a correlation between oxygen uptake and water loss in DDT-intoxicated P. regina. About 80 per cent of the total loss in weight was due to increased transpiration through the spiracles. They suggested that DDT causes a reduction in the hydrophobic properties of the spiracles. Dahm and Kearns (1951) attributed a significant loss in weight of DDT-poisoned houseflies to greater catabolic activity rather than to water loss.

The decrease of blood volume has been reported by a few workers. Roan et al. (1950) observed no apparent circulation of blood after four hours of poisoning with four different organophosphates, though the heart of Periplaneta americana (Linn.) contracted rhythmically. Chamberlain and Hoskins (1951) observed a similar phenomenon with parathion-poisoned roaches. Jochum (1956) attributed this phenomenon in parathion-poisoned silkworm larvae to the redistribution of available water. He found the gut of poisoned larvae to be filled with an unusually great quantity of fluid, which was less viscous than the blood. The hemolymph was also observed to be more basic, the pH rising from 6.8 to 7.7, while the gut fluid became less alkaline, the pH falling from 9.8 to 7.6. Changes in the specific gravity of the blood of intoxicated insects have also been reported and suggested as a criterion for prediction of toxicity of an insecticide (Patton 1962). Similarly, insects subjected to environmental stresses were found by Beament (1958) to suffer depletion of blood and to have increased the quantity of fluid in the hind gut.

The possibility of death through dehydration was studied by Krueger and O'Brien (1959), who concluded that the environmental humidity had no effect on the toxicity of malathion. The loss of weight or water

content from the body of insecticidally-intoxicated insects may itself not be important. However, the consequences, particularly the physical changes in the blood, accompanied by chemical changes such as accumulation of metabolites may prove hazardous.

2. Materials and Methods:

2.1 Materials:

The materials used during the present studies and their sources of supply are given on page 107 of the appendix.

2.2 Methods:

2.21 Rearing of Blattella germanica:

The roaches were reared in glass battery jars kept in an incubator at 30°C and 60-70 per cent relative humidity. The insects were supplied with water and rabbit pellets as food. Every month the food and water were changed and the jars were cleaned. During this process, the roaches were anaesthetised with carbon dioxide for five minutes.

2.22 Treatment of Roaches with Malathion:

Adult males, fifteen to twenty days old, were starved for eight hours (water was given) and released on a treated surface for forty minutes. The surface was prepared by spraying 10 ml of one per cent malathion in acetone (volume by volume from 95 per cent technical grade of malathion) on a piece of Whatman No. 1 filter paper 18 x 12 cm. This paper was then cut into a smaller rectangle 12 x 13 cm and two discs 4 cm diameter, to fit into a World Health Organisation test kit apparatus tube. The test apparatus

tube consisted of a plastic tube of 4 cm inner diameter and 12 cm long. One end of this tube was permanently closed by plastic netting while the other end had a screwable cap. The sprayed filter paper discs formed the treated surfaces at the two ends of the tube while the rectangle of treated filter paper lined the walls of the tube. Thus a total surface of about 181 square centimeters was provided.

2.23 Evaluation of Toxicity:

The treated roaches were released in clean observation jars, kept at 30°C and 50-60 per cent relative humidity, for seven hours without food or water.

Along with the treated groups, there was always a set of untreated controls.

Insects were then assigned to one of the following categories: hyperactive, locomotory activity higher than the controls; knockdown, flat on their backs and unable to regain an upright posture; paralysed, showing tremors of body parts; or dead, failing to respond to probing with a needle.

The studies to determine the time of knockdown of 50 per cent of the population (Kd. 50) were carried out by examining the treated roaches every 90 minutes after treatment, for six hours. However, in order to determine the threshold of knockdown, the insects were examined every 30 minutes for the first 90 minutes after treatment.

Observations of weight and blood volume were recorded every hour after malathion treatment, for six hours. For the blood volume studies, the treated roaches were separated into (a) unparalysed group, with roaches which were knocked down only after 5 hours of post-treatment time and (b)

paralysed group, with roaches that were knocked down during six hours of post-treatment period.

2.24 Collection and Measurement of Blood:

Sternburg's and Corrigan's method (1959) was modified to collect the blood in 15 ml graduated glass centrifuge tube. With the help of an oxygen-natural gas flame, a tube was pressed in at the 2 ml mark to form a concave constriction of uniform circumference. This constriction held a thin plastic ring which supported a glass wool filter.

Roaches were knocked down for about five minutes with carbon dioxide. Each individual was cleaned externally with a brush. The head and the complete alimentary canal of each roach was pulled out carefully by applying forceps at the neck. The process was facilitated by cutting off the end of the last abdominal segment. However, for quantitative amino acid and other biochemical studies, Sternburg's and Corrigan's method (1959) of sealing the mouth and anus with molten paraffin wax, was adopted. The legs of the roaches with sealed mouth and anus were clipped off and a small incision was made in the thorax along the pleuroventral lines.

Fifteen roach bodies with head and gut removed or with mouth and anus sealed, obtained from either of the two operations, were put in each modified centrifuge tube, with the posterior end upwards, on the glass wool filter and centrifuged for ten minutes at 200 g. Clear transparent blood collected at the bottom of the tube. The volume of the blood collected was read directly in the graduated centrifuge tube and confirmed by measuring with a micropipette.

2.25 Weight Loss:

In order to determine the loss of body fluid through defecation and regurgitation, both the poisoned and unpoisoned roaches were weighed after attaching ligatures of cotton thread around the neck and inter-segmental membrane of the last segment before the anus. The experiments were performed at rearing temperature and humidity. Insects were then dried to constant weight in an oven at 80°C.

2.26 Oxygen Uptake:

Six malathion-treated and six control roaches were put separately in Warburg flasks containing potassium hydroxide. The oxygen uptake was calculated according to Umbreit et al. 1959. The experiments were performed at 30°C.

2.27 Cholinesterase Inhibition Studies:

The activity of cholinesterase was measured manometrically by the use of a Warburg apparatus, on the malathion-treated and untreated control roaches of the susceptible and resistant strains. The method of Hartley and Brown (1955) was followed with slight modifications. The enzyme was prepared from brain tissues by homogenizing entire heads of 15 roaches in 26 mls of cold buffer pH 7.8. The buffer contained 0.025 M sodium bicarbonate, 0.04 M magnesium chloride and 0.15 M sodium chloride. The homogenate was centrifuged in cold at 500 g , to remove coarser particles. 0.007 M acetylcholine bromide was used as substrate.

Nine manometric flasks were used in each test of three replications. The thermobarometric flasks contained 3 ml of water, the enzyme flasks contained 2.6 ml of enzyme and 0.4 ml of substrate in the side arm and the

enzyme control flask contained 2.6 ml of buffer and 0.4 ml of substrate in the side arm.

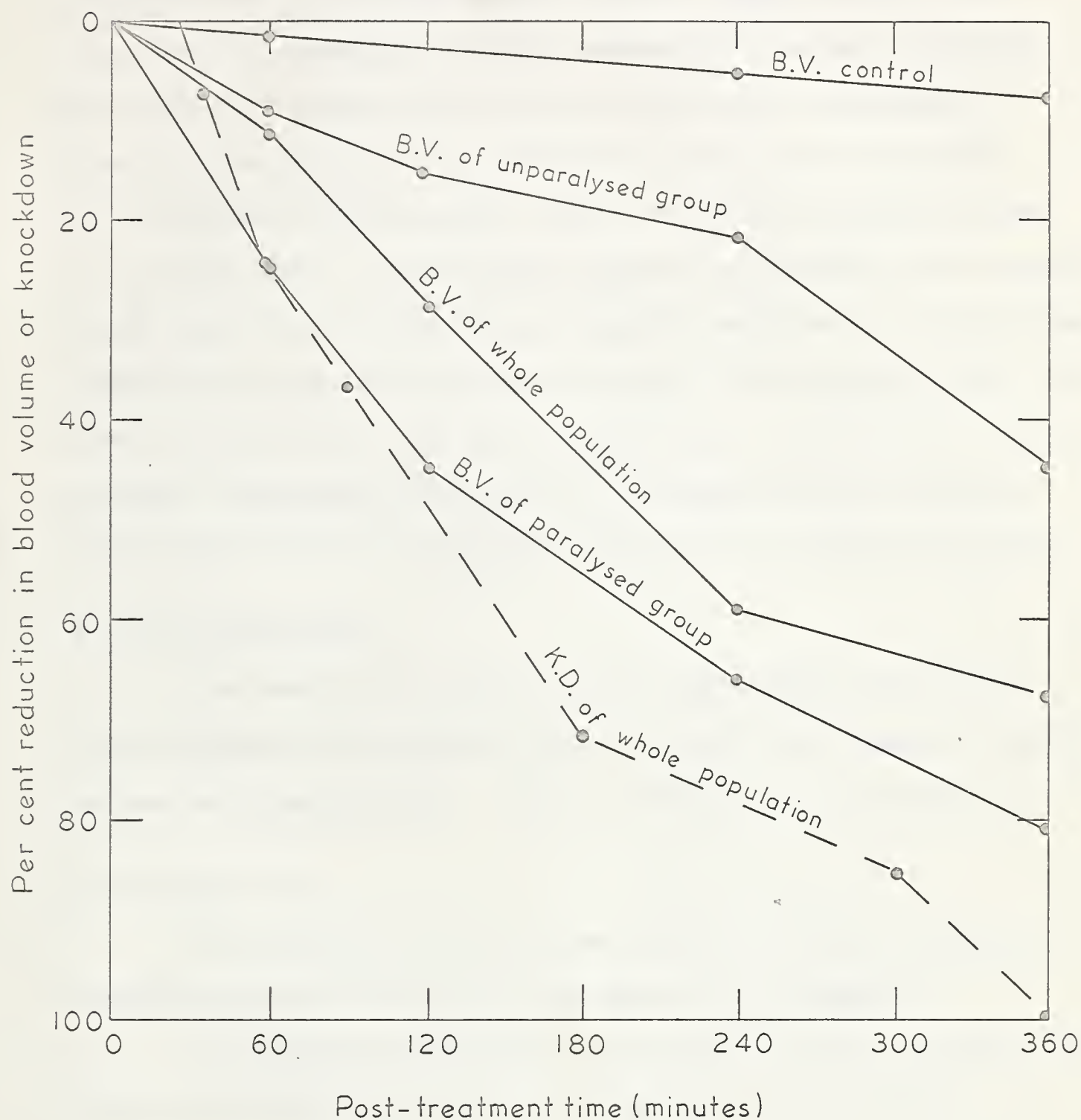
The atmosphere of the flask and the closed portion of manometer was replaced by a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide (Umbreit et al. 1959) and equilibrated at 30°C for 15 minutes. The substrate was then added to the enzyme by tipping the side arm. The gassing was repeated three times, followed by temperature equilibration. 30 minutes after the addition of the substrate to the enzyme, the stop-cocks were closed. Manometer readings were recorded at 10 minute intervals for the next hour.

3. Results and Discussion:

3.1 Results:

3.11 The Knockdown₅₀:

The results of the studies on the toxicity of malathion, with knockdown of the insects as criterion, are presented in Figure 1 and Table I of appendix. It may be observed that an initial period of about half an hour was required for the appearance of the toxic symptoms of knockdown. Once the threshold had been reached, the population was knocked down fairly rapidly and within the next 150 minutes as many as 75 per cent of the population were flat on their backs. Almost all the roaches were knocked down within six hours of treatment. Paralysed roaches were dead after about 6 - 8 hours of treatment. Based on the best possible straight line that could be drawn from the points on the graph in Figure 1, the Kd₅₀, or the time at which 50 per cent of the population was knocked down, was about 160 minutes.

Figure 1: Effect of malathion* on the knockdown and blood volume of *B. germanica*

* Dose lethal in seven hours ($0.46 \mu\text{l/sq. cm.}$)

K.D. = knocked down

B.V. = blood volume

3.12 The External Symptoms of Intoxication:

The external symptoms of poisoning differed with the post-treatment time. The treated insects initially showed a high rate of locomotion. As poisoning increased, hyperactivity was more pronounced and reached its maximum just before the roaches were knocked down. Almost all the individuals were observed running up the walls of the jar at the peak of hyperactivity. Later, they became incoordinated and fell on their backs. Sometimes they regained their posture once or twice before being finally knocked down. Paralysis then gradually took over and tremors of the legs were followed by tremors of the antennae and anal cerci. Finally the whole body would show violent tremors. At the onset of acute paralysis, longitudinal stretching of the abdomen occurred causing an exposure of the white intersegmental membrane and protrusion of the anus.

3.13 The Blood Volume:

The mean blood volume of 30 male adult roaches was 0.093 ml . Great differences were observed between the mean blood volumes of controls and the two poisoned groups. (Figure 1 and Table II of the appendix.)

3.14 Weight Loss:

The results of the loss in wet weight of poisoned roaches are presented in Tables III and IV of the appendix and in Figure 2.

In comparison with controls, the poisoned roaches had lost about 10 per cent of their dry weight, after six hours of intoxication.

3.2 Discussion:

The reduction in blood volume and loss in wet weight of the poisoned roaches were found to increase with progress in malathion intoxication. As a result of six hours of poisoning, the least intoxicated

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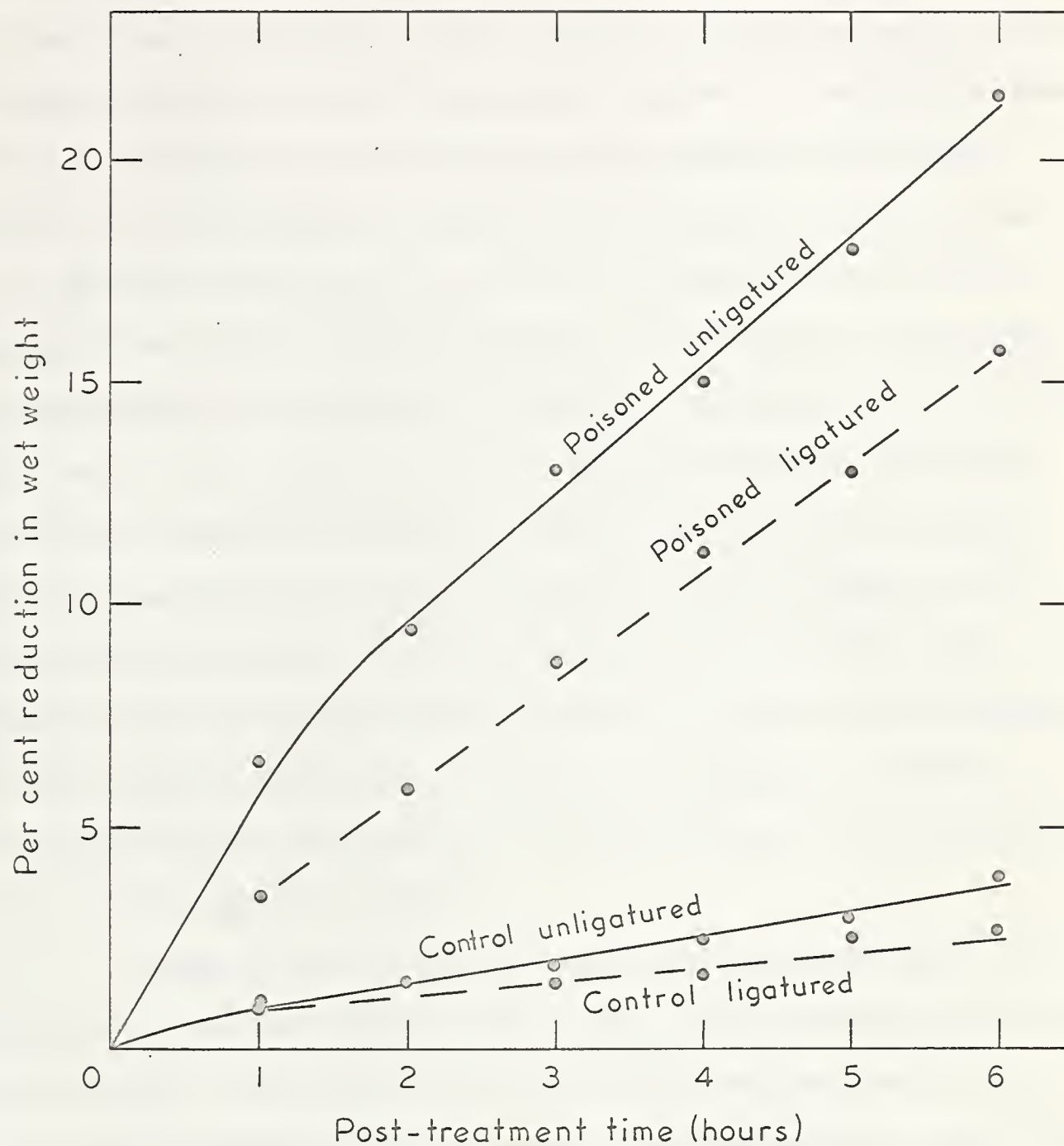
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Figure 2: Effect of malathion* on the loss of wet weight by male adults of *B. germanica*



* Dose lethal in seven hours ($0.46 \mu\text{l/sq. cm.}$)

unparalysed group of roaches lost about 36 per cent of the total blood volume. During the same period, the whole treated population and the most intoxicated paralysed group underwent about 60 and 72 per cent loss in blood volume, respectively. The correlation between the toxicity and the blood depletion is further evidenced by the rate at which the poisoned roaches were knocked down and the rate of the depletion of the blood. (Figure 1.) In the unparalysed group, the reduction was fairly gradual during the first four hours of intoxication. However, as the insects approached the knocked-down stage (between 5 and 6 hours in this group), the rate of loss of blood volume also increased and over 50 per cent of the total loss of blood volume had occurred during this period. The first four hours of poisoning knocked down about 80 per cent of the treated population, causing about 60 per cent depletion in the original blood volume of the population. Thus about 87 per cent of the water loss observed in the whole treated population after six hours of intoxication, had taken place during the first four hours of poisoning. A similar relationship between the knockdown of roaches and water loss was also observed in the paralysed group of roaches.

Disappearance of blood from American roaches poisoned with organo-phosphates was reported earlier by Roan et al. (1950) and Chamberlain and Hoskins (1951). Jochum (1956) also observed the same phenomenon in silk-worm larvae and explained it on the basis of redistribution of water. He suggested that the observed increase in the water content of the gut was at the cost of the hemolymph water content. During this investigation, it was observed that the fore and mid gut of the paralysed roaches were absolutely empty and dry, nor was extra water found in the hind gut. It

appears that different insects lose water in different ways. The depletion of the blood volume in malathion intoxicated German roaches was not caused by any noticeable redistribution of available water.

The depletion in blood volume was, in fact, the apparent result of the loss of water from the body. This, in turn, reduced the weight of the insects. The intoxicated roaches lost about 18 per cent of their wet weight during the six hours of poisoning. The increased catabolic activity, necessitated by the changed biochemical condition within the body, as evidenced by the hyperactivity and increased oxygen uptake (Figure 3 of the appendix) resulted in the utilization of reserve food.

The higher catabolic activity in the poisoned roaches is suggested by the 10 per cent loss in dry weight during six hours of intoxication. Increased oxygen uptake was showed in the malathion-treated German roaches during the first hour of intoxication, falling off gradually to the level of controls during the next hour and a half (Figure 3 of appendix). Harvey and Brown (1951) also observed similar effects of malathion poisoning to roaches. The loss in weight of DDT-treated houseflies was attributed by Dahm and Kearns (1951) to higher catabolic activity. As a result of DDT-intoxication, about a 90 per cent loss in glycogen reserves was reported in American roaches by Merrill et al. (1946) and in the Japanese beetle by Ludwig (1946).

The unligatured poisoned roaches were found to lose about 30 per cent more wet weight than the ligatured poisoned roaches (Figure 2). Thus the increased regurgitation and defecation in the poisoned roaches accounted for about one third of the loss in wet weight (mostly water) of the intoxicated roaches. Buck et al. (1952) observed only about 20

per cent loss weight in DDT-treated P. regina due to increased regurgitation while Jochum (1956) reported that all the wet weight lost by organophosphate poisoned silkworm larvae was the result of greater regurgitation. Malathion, by affecting the neurosecretory cells, could be responsible for increased contraction of the fore-gut, resulting in increased regurgitation in poisoned roaches. Clarke and Grenville (1960) demonstrated that the movement of the fore-gut of S. gregaria F. was controlled by the ingluvial ganglion.

It is thus obvious that 70 per cent of loss in wet weight was in the form of water, which was probably lost by increased transpiration through the spiracles and the intersegmental membranes and body wall that were observed to be stretched and exposed in the poisoned and paralysed roaches. The observed exposing and stretching of spiracles and intersegmental membranes could have been the result of failure of muscular control due to the expected accumulation of acetylcholine in roaches poisoned with an anticholinesterase. The majority of water loss in DDT intoxicated P. regina was also attributed to increased transpiration (Buck and Keister 1949).

Jochum (1956) suggested that the depletion of the blood in L. decemlineata was the cause of death after organophosphate poisoning. He also suggested that increased uptake of oxygen was the manifestation of water loss in parathion-poisoned insects. However, this hypothesis of death due to dehydration is open to question. The water content of the blood is important for normal transport of the essential materials. Any change in the physiological properties of the blood is likely to reflect upon the normal functioning of the systems closely related with the blood, such as pH and osmotic pressure. The consequence of physical

the : , of

changes in the blood are likely to prove fatal if accompanied by chemical changes too, such as the possible accumulation of nitrogenous waste. The reduction of blood alone is not likely to be fatal as many adult insects, particularly roaches, have been observed to feed and move normally, even though no blood was observed to be circulating (Patton 1962).

4. Conclusion:

Malathion intoxicated B. germanica showed initial hyperactivity followed by knock-down, paralysis, and death. The poisoned roaches lost both dry and wet weights. The 10 per cent loss in dry weight was due to utilization of reserve food. The original blood volume of the treated roaches was reduced by about 60 per cent.

The poisoned roaches lost about one-third of the total reduction in wet weight, through increased regurgitation and defecation. The remaining wet weight loss was mainly due to increased transpiration through spiracles and body wall. The malathion-treated paralysed group of roaches lost about twice as much blood volume as the unparalysed group. The intoxicated roaches, particularly the paralysed individuals, appeared to have lost muscular control which resulted in stretching and exposure of the spiracles and intersegmental membrane.

III THE FREE AMINO ACIDS OF THE HEMOLYMPH OF B. GERMANICA

1. Introduction:

The internal medium of no other animal group contains such a high concentration of free amino acids as insects. In 1944, Florkin suggested it as a special taxonomic character of insects (Florkin 1959). The usual amount of free amino acids in the blood of invertebrates (other than insects) is reported to be about 100 mg and of vertebrates, 50 mg per 100 ml of blood, (Wyatt 1961). In insects, the concentration of free amino acids increase with the evolutionary specialisation of the group (Florkin 1959).

About 20 common amino acids have been reported from the blood of insects. Of all the free amino acids present, glutamine, glycine, alanine, and proline and the basic amino acids, lysine, histidine and arginine are found to occur in highest amounts (Gilmour 1961). Many related compounds such as β -alanine, ornithine, α -aminobutyric acid, taurine, cystathione, 3-hydroxykynurenine, S-methyl cysteine and γ -aminobutyrate have also been reported by various workers in different insects (Wyatt 1961).

The concentrations of these amino acids are influenced by various factors such as temperature, food, starvation, stage of development, age, sex, and various pathological conditions (Wyatt 1961). Though the essential amino acids required by the insect group as a whole are the same as those of mammals, most of the insects synthesize all or most of the amino acids. Inclusion of arginine, lysine, tryptophan, valine,

alanine, proline, leucine and isoleucine, in the diet of German roaches has been reported to promote the growth, but very slightly (House 1949). However, all of the above mentioned amino acids except leucine could be synthesized by the insect. Auclair (1959) observed that when L-glutamate, L-glutamine, α -ketoglutarate, DL-alanine, DL-aspartic acid and L-asparagine were given individually in the diet to B. germanica, each of them affected the concentration of each of the above mentioned amino acids. Similarly, dietary experiments with L-cystine, β -alanine and DL-serine suggested an interrelationship among their metabolism. α -aminobutyrate, hydroxyproline, phenylalanine, and taurine, which are normally not detectable, could be detected after feeding a diet containing them. Methionine was found to give rise to α -aminobutyrate, while increasing the concentration of lysine and histidine in the blood of the German roaches.

The biochemical roles of these amino acids are not well known. Free amino acids do play an important part in maintaining the osmotic pressure of the blood (Buck 1953), in the transport of ammonia (Gilmour 1961), and in the production of energy via Krebs TCA cycle (Winteringham and Harrison 1956).

2. Materials and Methods:

2.1 Materials:

The materials used in this investigation and their sources are given on page 107 of the appendix. The following reagents were prepared in the laboratory.

2.11 The Standard Solutions of Amino Acids:

The standard solutions of the known amino acids were prepared by dissolving the amino acids in water so as to give 0.1 micromoles of amino acids per 0.1 ml of solution. For drawing the standard curve for quantitative studies, at least four concentrations, ranging from 0.05 to 0.2 micromoles (up to 0.50 micromoles in the case of glycine) were prepared in the above mentioned manner. Tyrosine was dissolved as sodium salt (using sodium hydroxide).

2.12 Electrophoresis Buffer and Chromatography Solvents:

(i) Electrophoresis Buffer pH 2.2:

One litre of the buffer solution contains 3 ml of pyridine and 40 ml of formic acid and 957 ml of deionized water (Richmond and Hartley 1959).

(ii) Butanol-butyl Acetate Solvent:

n-butanol, butyl acetate, acetic acid, and water were mixed in a ratio of 19 : 1 : 5 : 25 . The solvent was prepared in a separating funnel and the lower layer (water) was used to saturate the atmosphere in the chromatocab. (Richmond and Hartley, 1959)

(iii) Phenol pH 12:

50 ml of 0.067 M Na_2HPO_4 and 50 ml of 0.067 M NaOH were added to 100 ml of phenol (McFarren 1951).

(iv) Collidine Solvent:

The collidine solvent was prepared by mixing 2-6 lutidine, 2-4-6 collidine, water and diethylamine in 100 : 100 : 100 : 3 ratio. (Block 1958.)

(v) Phenol-water Solvent:

100 mls of de-ionized water was dissolved in 400 mls of phenol by gentle warming. To this was added 25 mg of 8-hydroxyquinoline. The solution was stored, cold, in a dark bottle. Before use, the bottle was shaken thoroughly, warmed and the required quantity taken out. A beaker containing 100 mls of 0.3 per cent NH_4OH was kept in the chromatocab (Block 1958).

2.13 Acetate Buffer:

The method of Moore and Stein (1954) was modified for greater purity. To 320 grams of anhydrous NaOH, enough water was added to make the volume up to 1.1 litres. This was done on a cold water bath. To this solution was added very slowly and with constant stirring, 670 ml of concentrated acetic acid. The pH obtained was 5.51 ± 0.03 . The total volume was then brought with water to two litres. The buffer was stored at 4°C .

2.14 Hydrindantin:

The compound was prepared in the laboratory by the method described by Connell et al. (1955). Ten grams of ninhydrin was dissolved in 250 ml 0.5 N acetic acid, at 30°C . Five grams of ascorbic acid was dissolved in this solution, which was warmed to 65°C with constant stirring, when a copious crop of white hydrindantin crystals appeared. After leaving it for ten minutes at this temperature, two grams of ascorbic acid was added and the temperature maintained for another five minutes. This solution was now cooled rapidly and filtered on a sintered glass funnel, washed twice with distilled water and dried in vacuum over sulphuric acid.

2.15 Borate Buffer:

9.28 gm of boric acid was dissolved in a small volume of methanol. To this was added 32.5 ml of 6 N KOH and the solution made up to 500 mls with methanol. This gave an approximate pH of 11.5 (Tigane et al. 1961).

2.16 Ninhydrin Detection Spray:

The spray was prepared by adding 50 mg of ninhydrin to 75 ml of absolute ethanol and 25 ml of 2 N acetic acid.

2.17 Detection Reagent Solution:

Modified ninhydrin-hydrindantin reagent was prepared according to Matheson et al. (1961). Due to the instability of this reagent, only 400 ml were prepared at a time. 1.52 gm of ninhydrin was dissolved in 228 ml of methyl cellosolve. To this solution was added 72 ml of water and 100 ml of acetate buffer, pH 5.5. The bottle was degassed by being held under vacuum for 30 minutes on a magnetic stirrer. Nitrogen was then flushed through the solution for 30 minutes, followed by addition of 0.24 gm of finely powdered hydrindantin to the solution. Nitrogen was again flushed for 30 minutes while the magnet continued to stir. The whole preparation was carried out at 4°C. The reagent solution was checked every time before use by centrifuging a small volume and adding 4 ml of the supernatant to 2 ml of 8 M NaOH. The absorbancy of this "hydrindantin blue" should be between 0.4 and 0.5 at 570 mμ.

2.2 Method:

2.21 Collection of Blood Sample:

The blood samples were collected from laboratory reared cultures of susceptible and resistant strains of B. germanica. Fifteen to twenty days old male adults were deprived of food but not water, for eight hours before the blood was collected by the method already described on page 9. Each blood sample was taken from 30 males of one strain.

2.22 Isolation of Amino Acids:

The free amino acids were isolated from the blood by precipitating out blood protein after the method of Auclair (1950). 2 ml of cold 90 per cent ethanol was added to the blood, shaken thoroughly and centrifuged for ten minutes at 200 g in an International Clinical Centrifuge. The protein settled down at the bottom and the supernatant with amino acids was decanted. The residue was washed four times, each time with 0.5 ml of cold 90 per cent ethanol, followed by five minutes of centrifugation and decantation. All these operations were carried out at 4°C. The supernatant was then dried in vacuo. The amino acids were redissolved in 0.1 ml of water and spotted on the filter paper.

2.23 Separation of Amino Acids:

Richmond and Hartley's (1959) method of a two dimensional system of high voltage electrophoresis followed by paper chromatography was adopted. Electrophoresis was done at pH 2.2 under toluene and at 2100 volts for 90 minutes. The descending chromatography was done in butanol-butyl acetate solvent for 16 to 18 hours.

On a 58 x 20 cm Whatman No. 3, MM filter paper, the amino acid spots were applied four centimeters apart and fifteen centimeters from

the bottom (anode). Four spots could thus be applied to one paper. The paper was then wet with buffer pH 2.2, taking care that the spots were not drained, and subjected to electrophoresis. The paper which now contained amino acids in a strip, upto about 35 cm from the origin, was dried in a hood. Such strips, 3 x 38 cm were sewn length-wise on both the sides, to a slightly smaller window which was previously cut out in another sheet of paper. This window was made about seven centimeters from the top of 42 x 48 cm filter paper. Each paper was then subjected to chromatography in the second dimension.

It was difficult to separate alanine and β -alanine, histidine and arginine and, at times, glutamate and threonine. Descending, one-dimensional chromatography, in phenol pH 12 gave a very good separation of glutamate and threonine. For other amino acids a two dimensional system of phenol (first phase) and collidine was employed.

2.24 Identification of the Amino Acids:

The air-dried chromatogram was sprayed with ninhydrin detection spray with the help of a glass atomizer. The rate of spraying was about one millilitre per hundred square centimetres. The spots were developed by keeping this paper in an oven at 65°C for about 10 minutes. The spots of the chromatogram of the blood sample were identified by comparing the R_f values of each spot with the R_f values of the known standard amino acids. The latter were obtained for each amino acid separately and in mixture with all other amino acids known to be present in the unknown, for each of the solvent systems described above. To be always sure of the position of each spot, each set of the unknown sample was run together

with a known mixture of the amino acids.

2.25 Quantitative Estimation of Amino Acids:

The method of Connell et al. (1955) was followed. Each spot was delineated with the help of a pencil and a plastic template. An equal sized spotless paper was cut from the vicinity of each spot to serve as blank for that spot. Each spot and its blank was held in a pair of forceps and soaked with 0.2 - 0.3 ml of borate buffer and dried in a miniature air tunnel. Soaking with the borate buffer and drying was repeated twice. Spots and blanks were then cut into small pieces and placed in a tube with 10 ml markings. Nitrogen was then flushed into each tube for ten minutes. Two millilitres of the detection reagent was added in each tube. The tubes were then covered with aluminium caps and kept in a boiling water bath for twenty minutes and cooled immediately under running water. Spots were eluted by adding 50 per cent ethanol and bringing the volume of each tube to the 10 ml mark. Each tube was shaken thoroughly then centrifuged at a low speed for five minutes to allow the pieces of paper to settle down. The supernatant solution was then read for absorbancy in a Beckman DU Spectrophotometer. Except for proline which was read at 440 m μ , all other solutions were read at a wavelength of 570 m μ . Cells of 1 cm path length were used.

The concentration of the amino acid in each spot was estimated quantitatively by reading the absorbancy against the concentration in the standard curve of that amino acid. At least four mixtures containing the known amino acids in four different known concentrations were prepared. Chromatograms were developed and the absorbancy of each spot was read in

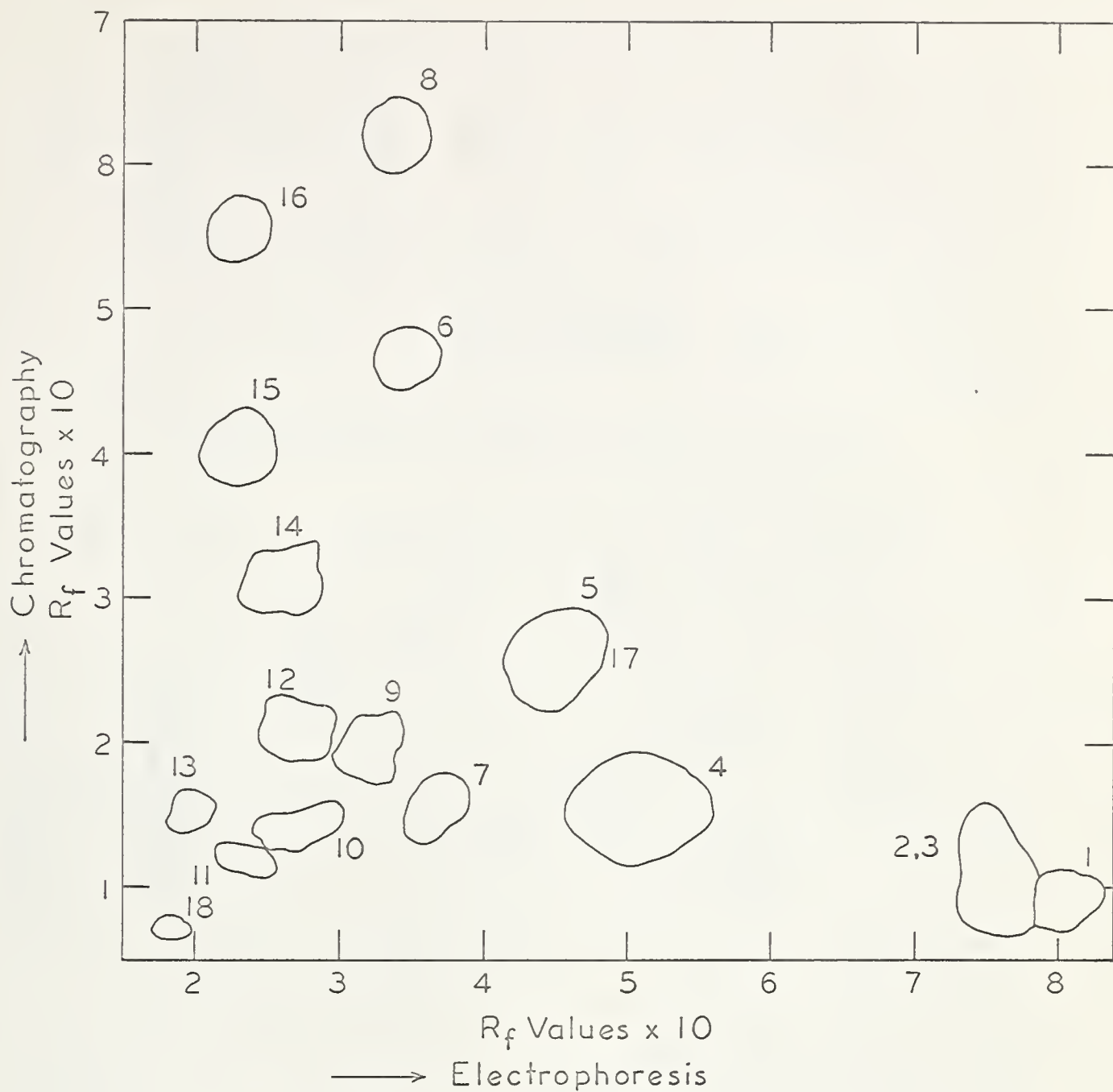
the usual way. Such standard curves were plotted for nineteen amino acids. Except for β -alanine, histidine, and arginine, all other standard curves were prepared by developing the chromatograms after subjection to the electrophoresis and butanol-butyl acetate chromatography. Concentrations of α and β alanine were calculated by subtracting the concentration of β -alanine from the alanine concentration determined after electrophoresis. Standard curves for alanine spots were made with mixture of α and β alanine in equal amounts. Standard curves for glutamate, threonine, arginine and histidine were prepared after running the chromatograms in the solvent system used for their separation. Standard curves for glutamine and asparagine were also prepared for the phenol collidine solvent system. The reproducibility of the standard curves for amino acids was within fifteen per cent.

3. Results and Discussion:

3.1 Qualitative Studies:

The results of qualitative studies are presented in Figures 3 and 4. Based upon the R_f values of known compounds (Table V of appendix), eighteen amino acids were qualitatively separated from the blood of B. germanica, by the two dimensional system of high voltage electrophoresis and chromatography. The amino acids identified were lysine, histidine/arginine, glycine, alanine, valine, serine, leucine/isoleucine, threonine, glutamine, asparagine, glutamate, aspartate, proline, tyrosine, methionine, and cystine. Histidine, arginine, α -alanine, β -alanine, asparagine and glutamine were further identified by separating in phenol-collidine two dimensional system (Figure 4).

Figure 3. Separation of amino acids of the blood of *B. germanica* by electrophoresis and chromatography.



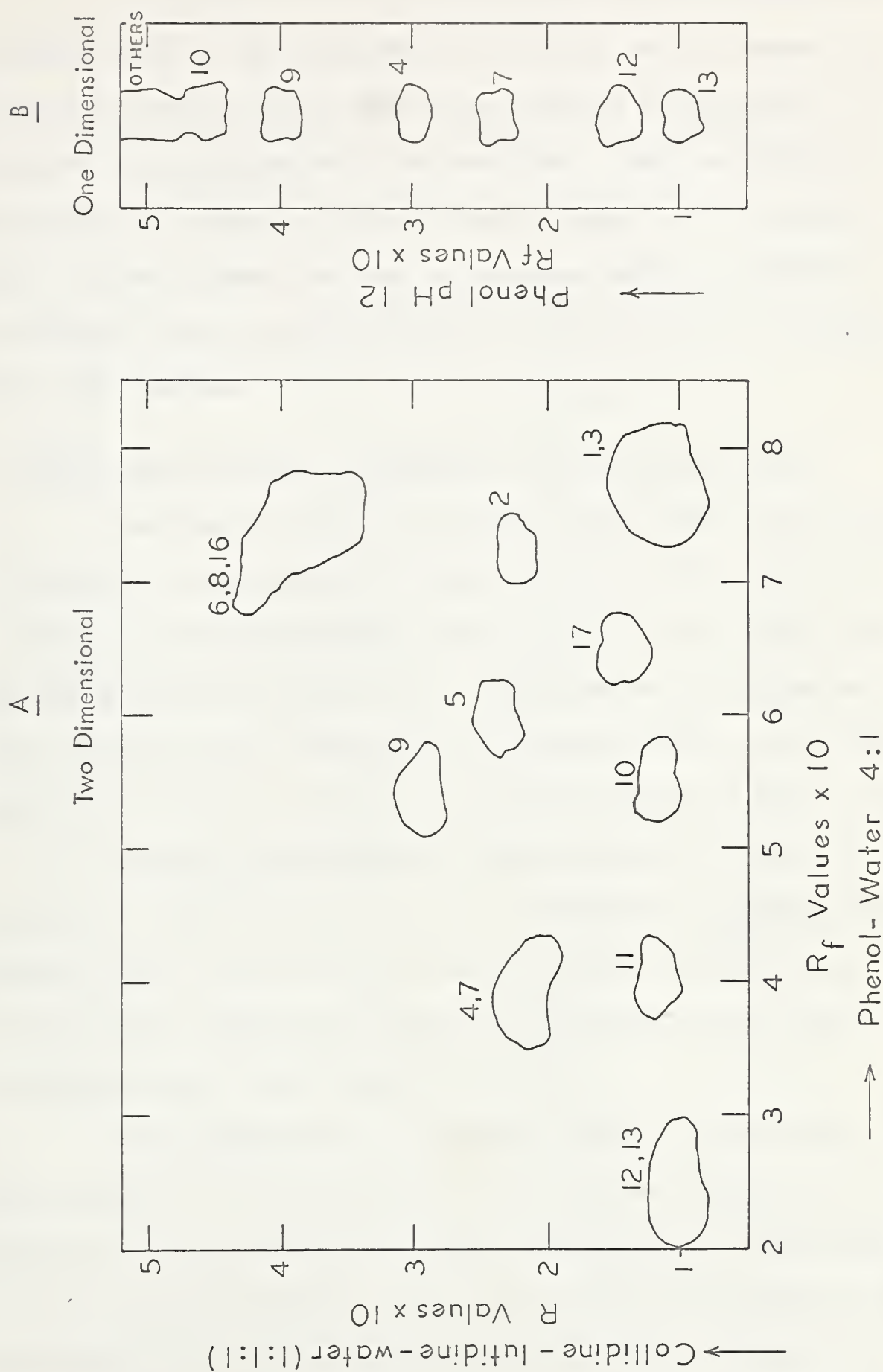
LEGEND

1	Lysine	11	Asparagine
2	Histidine	12	Glutamate
3	Arginine	13	Aspartate
4	Glycine	14	Proline
5	Alanine	15	Tyrosine
6	Valine	16	Methionine
7	Serine	17	β - Alanine
8	Leucine/Isoleucine	18	Cystine
9	Threonine		
10	Glutamine		

Electrophoresis: pH 2.2 at 2100 volts for 90 minutes

Chromatography: n-butanol-butyl acetate-acetic acid-water (19:1:5:25)

Figure 4. Separation of amino acids of the blood of *B. germanica* by descending chromatography.



Legend: As in Figure 3.

Pratt (1950) reported the presence of the above mentioned 18 free amino acids in the hemolymph of the German roach. However, his qualitative studies did not confirm the presence of histidine as its separation from arginine was never consistent. The same difficulty was experienced during the present studies though not to the same extent. In cases where histidine and arginine were not separated, the ninhydrin colour was almost equal to the sum of the two separate spots, when obtained.

3.2 Quantitative Studies on Susceptible and Resistant Strains:

Quantitative studies on the free amino acids of the blood of both resistant and susceptible strains of German roaches were carried out. No quantitative differences were observed in any of the free amino acids between the two strains. Hence the results presented in Table 1 represent the concentrations of the free amino acids of the blood of the resistant as well as susceptible strains of German roaches.

The total concentration of free amino acid was found to be 246.98 mg per 100 ml of blood. Another orthopteran, Carausius morosus contained about 300 mg of amino acids per 100 ml of blood (Gilmour 1961). Thus the German roach has the lowest concentration of free amino acids in the blood of any insect reported thus far.

The concentrations of different amino acids show great variation among themselves. There was a 22.5 fold difference between the largest (glycine) and the smallest concentration (methionine). Though there were 18 amino acids present in the blood, glycine alone formed about 20 per cent of the size of the free amino acid pool. Proline (about 9 per cent) and glutamine (about 11 per cent) along with glycine

Table I

Free amino acids of the hemolymph of B. germanica ,
susceptible and malathion resistant strains.

Amino acids	Concentrations of amino acids		
	micromoles per 93 μ l.		mg. per 100 ml.
		<u>S. E.</u>	
Glycine	0.60	± 0.018	71.95
Glutamine	0.180	± 0.009	28.28
Proline	0.175	± 0.016	21.44
Lysine	0.080	± 0.006	15.71
Arginine	0.060	± 0.007	13.68
Alanine	0.130	± 0.008	12.44
Tyrosine	0.055	± 0.002	10.71
Asparagine	0.075	± 0.005	10.66
Glutamate	0.050	± 0.004	7.91
Cystine	0.030	± 0.0016	7.74
Valine	0.060	± 0.0018	7.56
Leucine/isoleucine	0.050	± 0.0018	7.05
Serine	0.060	± 0.006	6.78
Histidine	0.030	± 0.0045	6.75
β -alanine	0.070	± 0.007	6.78
Aspartate	0.035	± 0.0025	5.01
Threonine	0.025	± 0.003	3.20
Methionine	0.02	± 0.0008	3.20
Total	17.62		246.98

Blood sample collected from 30 male adults.

constituted about half of the amino acid pool of the blood. The acidic groups of aspartate and glutamate were in a very low concentration of about 5.23 per cent of the total. The basic amino acids, lysine, histidine and arginine were comparatively in higher amounts, contributing 14.6 per cent towards the amino acid pool. Thus the major portion (about 80 per cent) of free amino acids in the blood was the neutral group.

The high concentration of glycine might suggest the important role of this amino acid in the physiology of the insect. The major end product of glycolysis in insects is α -glycerophosphate and the production of pyruvate is slower than in mammals (Chefurka 1959). Under these circumstances it would not be surprising to expect the insects to synthesize this amino acid from a point early in the degradation of carbohydrates. Insects would then follow the mammalian sequence of reaction for the synthesis of serine from 3-phosphoglycerate, via 3-phosphopyruvic acid and phosphoserine. Glycine could then be synthesized from serine. The major pathway of glycine catabolism could then be expected to be pyruvate via serine.

The low concentration of glutamate and aspartate may be due to the ability of the insect to convert glycine and a large number of other amino acids to glutamate by transamination mechanism known to occur in insects (Kilby and Neville 1957, Bheemeshwar 1959).

The high aspartate-asparagine ratio of 1:2 and the glutamate-glutamine ratio of 1 : 3.58 suggest a significant role of these amino acids in the mechanism of ammonia transportation within the body. Treherne (1960) has shown a high glutamate and glutamine ratio within

the nerve cord (central nervous system) of the American cockroach. Glutamate and glutamine would be expected to play an important role in maintaining the pH of the physiological fluid. It would also then be expected that proline, the amino acid present in the third largest concentration plays an important indirect role in ammonia transport by supplying the substrate for the synthesis of glutamine and urea.

The biochemical significance of such a high concentration of free amino acids in the blood of B. germanica as compared to other invertebrates, is rather difficult to decide. However, the maintenance of osmotic pressure and pH, the synthesis of protein and urea and the provision of a readily available substrate for the Krebs cycle are some of the roles these amino acids may play.

IV THE EFFECT OF MALATHION ON AMINO ACID METABOLISM IN SUSCEPTIBLE AND RESISTANT STRAINS OF B. GERMANICA

1. Introduction:

The insect hemolymph is characterised by the presence of large quantities of free amino acids. The biochemical roles of these amino acids are not well known. Hill (1962) has reported synthesis of protein from free amino acids under the influence of neurosecretory cells. He has also reported that free amino acids of the blood are in equilibrium with the blood protein. Some of the free amino acids such as glycine, glutamate, alanine, and phenylalanine have been observed to take part in the synthesis of silk by silkworms (Florkin 1959). The role of free amino acids as a readily available substrate for Krebs' TCA cycle has also been suggested (Winteringham and Harrison 1956, Corrigan and Kearns 1963).

The general pathways of amino acid metabolism in insects are probably similar to those of mammals (Gilmour 1961). Kilby and Neville (1957) have shown homogenates of fat body of the desert locust to be able to synthesize glutamate from α -ketoglutarate by the transfer of an amino group from any one of alanine, glycine, L-aspartate, L-leucine, L-valine, DL-serine, DL-threonine, DL-phenylalanine, L-tyrosine, L-lysine, L-tryptophan, L-histidine, L-ornithine, L-argine, L-cystine, L-cysteine and L-methionine, but not from proline or hydroxyproline.

Kilby and Neville (1957) also observed oxidative deamination of L-amino acids in the fat body preparations. However, they thought

the reaction to be due to transamination of α -ketoglutarate followed by dehydrogenation of glutamate thus formed.

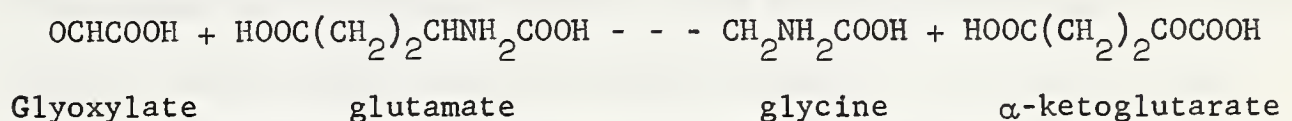
The presence of glutamic dehydrogenase, linked to the cytochrome system has been demonstrated in the desert locust (Kilby and Neville 1957).

Bheemeshwar (1959) has shown β -decarboxylation of aspartate to form α -alanine in the silk glands and the gut of the silkworm, with the release of carbon dioxide.

Glycine Metabolism:

Only a few of the complex reactions of glycine metabolism have been studied in insects. The possible synthesis of glycine from 3-phosphoglycerate has already been discussed (page 32). In mammals, glycine may be metabolised to creatine, protoporphyrin, purines, glutathione, glyoxylic acid, serine and hippurate and some other compounds shown in Figure 4 of the appendix.

Fukuda and Hayashi (1958) demonstrated transamination of glycine to glutamate and glyoxylic acid in the silk glands of Bombyx in the following manner:



Conversion of glycine into serine has been shown by McEnroe and Forgash (1957) in P. americana. Bricteux-Gregoire et al. (1958) observed the incorporation of glycine $(2)\text{C}^{14}$ into alanine in silkworms. This incorporation was probably achieved through pyruvate as an intermediate. Formation of hippuric acid from glycine has also been shown

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in insects (Friedler and Smith 1945).

Proline, Glutamine and Glutamate Metabolism:

The metabolism of these amino acids has not been studied in insects. Winteringham and Harrison (1956) reported incorporation of acetate (2) C^{14} into proline, glutamine and glutamate in houseflies. The incorporation of labelled proline into glutamine and glutamate has also been reported (Corrigan and Kearns 1963). Kilby and Neville (1957) have shown the conversion of glutamate to glutamine in the fat body homogenates of desert locust. These observations suggest a mammalian-like interrelationship in the metabolism of proline, glutamine and glutamate. Proline can be converted into glutamate, glutamine as well as α -ketoglutarate, a substrate for Krebs TCA cycle (Figure 5 of the appendix).

Effect of Insecticides on Amino Acid Metabolism:

The effects of a few chlorinated hydrocarbons, two organophosphates and some of the pyrethrins, on the free amino acids of the blood of five different species of insects have been studied by Winteringham and Harrison (1956), Joseph (1958) and Corrigan and Kearns (1963). There appears to be no general pattern to the way in which insecticides affect free amino acids. Insecticides of the same group i.e. DFP and TEPP (organophosphates) have different effects on the same insect. On the other hand, TEPP and dieldrin and DFP and DDT, belonging to two different groups of insecticides have similar effects on the free amino acids of the same insect.

Ludwig and Bartolotta (1953) compared the blood of normal Popillia japonica (Newman) with those of DDT intoxicated and starved beetles. In the DDT intoxicated beetles, they reported increases of about 56 per cent in non-protein nitrogen, 75 per cent in amino nitrogen, 78 per cent in urea nitrogen and about 26 per cent in reducing compounds. Similar effects were observed in the blood of starved beetles. This led them to suggest an analogy between DDT action and starvation. Joseph (1958) studied the blood of normal and DDT poisoned Tenebrio molitor (Linn). He noted that the intoxication caused increases of 2 per cent in protein nitrogen, 50 per cent in non-protein nitrogen, 90 per cent in reducing compounds and 70 per cent in amino nitrogen. He also observed significant increase in almost all the amino acids. Only two compounds, taurine and norleucine registered a decrease, in each case of about 30 per cent. The amino acids that increased the most were alanine (400 per cent), proline (159 per cent), histidine (144 per cent) methionine (122 per cent). Joseph supported the suggestion of Ludwig and Bartolotta that starvation which follows DDT intoxication brings about death in poisoned insects. Roan and Hopkins (1961) have linked these observations with the depletion of carbohydrate reserves.

Other workers, however, have shown that DDT intoxication in B. germanica, P. americana and Musca domestica (Linn.) does not affect any amino acid except proline which is decreased. Corrigan and Kearns (1963) have shown a selective correlation between the appearance of symptoms of DDT poisoning in American roaches and the disappearance of proline. When intoxicated insects were kept at a lower temperature,

the toxicity of DDT increased and the symptoms appeared in greater severity. This resulted in rapid disappearance of proline from the blood. However, proline reappeared when the symptoms were alleviated by bringing the insects to a higher temperature. Hoy and Gordon (1961) have observed about 75 per cent depletion of free proline in the body of DDT poisoned German roaches. Injection of a large dose of proline was observed to have no toxic effects. They reported about 50 per cent more utilisation of injected proline in DDT poisoned roaches than in controls. Corrigan and Kearns (1963) also observed proline depletion in DDT treated tobacco hornworms and houseflies.

On the basis of reduction in the concentration of α -keto-glutarate in the blood of DDT intoxicated roaches, Corrigan and Kearns (1963) have suggested some block in the availability of a substrate to Krebs' TCA cycle, above the α -ketoglutarate level. Their findings are in agreement with the observations of Agosin et al. (1961) that some glycolytic enzymes are inhibited in DDT treated Triatoma infestans (Leconte). They thus suggest the utilization of proline by Krebs' TCA cycle.

Pyrethrins and lindane were found to cause only a slight decrease in proline while dieldrin and TEPP produced no change in the concentration of any amino acid of the blood of American roaches (Corrigan and Kearns 1963).

Winteringham and Harrison (1956) studied the effect of DDT and DFP poisoning on the incorporation of acetate (2) C^{14} into various free amino acids in houseflies. Both the insecticides are reported

to have a similar effect on the free amino acids. The DFP treated and untreated controls were observed to have about 75 per cent of the activity of total soluble fraction incorporated into glutamine, glutamate and proline. However, the proportions of these amino acids were different in the two sets of flies. Five hours after administration of labelled acetate, glutamate-proline and glutamine were found to constitute 57.4 and 18.6 per cent respectively, of the total soluble fraction in the controls. During the same time, in DFP treated flies, glutamine increased to 45 per cent whereas glutamate-proline fraction was decreased by about 30 per cent. Free alanine and aspartic acid had incorporated only 3 and 1.5 per cent of the total activity respectively. No effect of DFP was found either on the levels of adenosinetriphosphate or on phosphorylated intermediates which could release ammonia. Winteringham (1959) thus concluded that DFP action causes deamination of proline, the free ammonia being trapped by glutamate to form glutamine. He further added; "These observations are consistent with the increased utilization of free amino acids via Krebs' TCA cycle following deamination, the resulting ammonia being trapped, temporarily at least, as glutamine. Significant increases in glutamine however, have only been demonstrated under abnormal conditions of insecticidal action ."

Corrigan and Kearns (1963) also observed slight increase in free glutamine of DDT poisoned roaches. However, they thought the increase to be too small and insignificant, when compared with the amount of administered proline utilized by the intoxicated roaches.

McAllen and Brown (1960) studied the effects of various insecticides on the transaminases of American roaches. DDT, endrin, toxaphene and sodium ortho-arsenate were found to have no in vivo effects while malathion appeared to increase the activity of the enzyme complex.

Thus the reduction of free proline in the DDT, DFP and TDE intoxicated insects has been established. However, the fate of ammonia released by deamination of amino acids is not known. Ammonia toxicity to poisoned insects remains a possibility. It is also not clear if the depletion of amino acids is a result of direct or indirect action of the insecticides.

2. Materials and Methods:

2.1 Materials:

The materials used during the study and their sources of supply are given on page 107 of the appendix.

2.2 Methods:

2.21 Determination of Free Amino Acids of the Blood:

To study the effect of malathion on the free amino acids of the blood, both the malathion resistant and susceptible strains of B. germanica were used. Both strains of roaches were treated with malathion in a similar manner (page 7). The blood collection and the quantitative estimation of amino acids was done according to the procedure described on page 9. Thirty roaches were used in each experiment and all experiments were repeated at least three times.

The treated roaches of both strains were divided into three categories as follows:

1. The resistant group: consisting of roaches of the malathion-resistant strain only.
2. The susceptible paralysed group: consisting of roaches of the susceptible strain that were knocked down and paralysed within ninety minutes of malathion treatment.
3. The susceptible unparalysed group: consisting of susceptible strain roaches which showed no external symptoms of toxicity except for hyperactivity, and the roaches that were paralysed only after 270 minutes of malathion treatment.

For the quantitative estimation of free amino acids, blood samples of thirty roaches were drawn from each of the treated groups, at 90, 180, 270 and 330 or 360 minutes after malathion treatment. It was impossible to obtain a quantitative collection of blood from the paralysed group of the susceptible strain after 360 minutes of intoxication. Thus the last sample from this group was drawn 330 minutes after malathion treatment. The resistant strain never contained more than 7 - 10 per cent of paralysed individuals in any sample (Figure 1 of appendix).

2.22 Determination of Nitrogen and Urea of the Blood:

Total nitrogen and protein and non-protein nitrogen analyses of the blood of both malathion treated and untreated roaches were carried out by the micro-Kjeldahl technique (Street et al. 1946).

Blood urea was determined according to the method described

by Umbreit et al. (1959). Blood was collected from sixty cockroaches after five and a half hours of malathion. The volume was measured and brought to 2.5 ml by adding acetic acid-sodium acetate buffer, pH 5. After centrifugation at 300 g for five minutes, 2.4 ml of the supernatant was taken in a Warburg flask with 0.5 ml. of urease solution (10 mg per ml) in the side arm. The control vessel contained distilled water in place of urease solution. 0.1 ml of aniline was also added to the main compartment of the flask. Thus a volume of 3 ml of solution was maintained in every flask. After about ten minutes of equilibration time at 30°C, the solutions were mixed by tipping the side arm and after a couple of minutes, the stoppers were closed and readings recorded for the next thirty minutes.

3. Results and Discussion:

3.1 Results:

The effects of malathion on the concentrations of free amino acids of susceptible and malathion resistant strain of German roaches are presented in Tables II-A, II-B and Figures 5 and 6.

3.11 Depletion of the Amino Acids:

Malathion intoxication caused a reduction in the total concentrations of the free amino acids of the blood of both the resistant and susceptible strains. The paralysed and unparalysed groups of the susceptible strain were observed to lose about 38 and 31 per cent respectively of their total free amino acids, in 330 minutes of malathion

Table II-A

The effect of malathion* on the concentrations¹ of free amino acids of the hemolymph of B. germanica (susceptible strain).

Amino acids	Control	Post-treatment time (minutes)								Total loss	
		90		180		270		330			
		SP	UP	SP	UP	SP	UP	SP	UP	SP	UP
Glycine	0.60 ± 0.018	0.35	0.42	0.24	-	0.20	0.30	0.18 ± 0.01	0.22 ± 0.01	0.42	0.38
Proline	0.175 ± 0.016	0.13	0.14	0.085	-	0.055	0.11	0.03 ± 0.001	0.65 ± 0.002	0.145	0.11
Glutamate	0.05 ± 0.004	0.03	0.04	0.025	-	0.02	0.03	0.01 ± 0.001	0.017 ± 0.001	0.04	0.033
Glutamine	0.18 ± 0.01	0.155	0.165	0.125	-	-	0.145	0.06 ± 0.006	0.10 ± 0.01	0.12	0.08
Others	No change	at any time									
* Dose lethal in seven hours (0.46 µl/sq.cm)		SP		Paralysed group							
1 Micromoles per 30 male adults		UP		Unparalysed group							

Table II-B

Effect of malathion* on the concentrations¹ of free amino acids of the hemolymph of B. germanica (resistant strain).

Amino acids	Control	Post treatment time (minutes)				Total loss
		90	180	270	360	
Glycine	0.60 ± 0.018	0.50	0.50	0.50	0.50 ± 0.0035	0.10
Proline	0.175 ± 0.016	0.13	0.13	-	0.155 ± 0.006	0.02
Glutamate	0.050 ± 0.004	0.040	0.045	0.045	0.045 ± 0.0027	0.005
Glutamine	0.180 ± 0.009	0.185	0.180	0.180	0.185 ± 0.013	-
Others	No change at any time					

* Dose lethal in seven hours (0.46 µl/sq. cm.)

¹ Micromoles per 30 male adults

poisoning. The total concentration in the paralysed group was found to drop down to about 154 mg from the original concentration of 249 mg of free amino acids per 100 ml of blood. The unparalysed group was found to contain about 170 mg and the least intoxicated resistant group, about 232 mg per 100 ml of blood.

The depletion of the total concentration was caused by the reduction in the concentrations of glycine, proline, glutamate and glutamine. The poisoned resistant strain showed reduction of glycine, glutamate and proline. The concentrations of the remaining fourteen free amino acids were the same in poisoned roaches as in the unpoisoned controls.

3.12 Changes in Blood Urea and Nitrogen:

Normal German roaches were observed to contain 520 mg of protein, 275 mg of non-protein and 770 mg of total nitrogen per 100 ml of blood. After five and a half hours of malathion intoxication, with a dose-lethal in seven hours, protein nitrogen had increased to 540 mg, non-protein to 428 mg and total nitrogen to 1070 mg per 100 ml. Thus the poisoned roaches of the susceptible strain showed an increase of about 55 per cent in the non-protein and about 40 per cent in the total nitrogen. The blood urea contents of the poisoned roaches also increased by about 44 per cent: from 26.3 mg in the controls to 37.8 mg per 100 ml.

3.2 Discussion:

Malathion Toxicity and Amino Acid Metabolism:

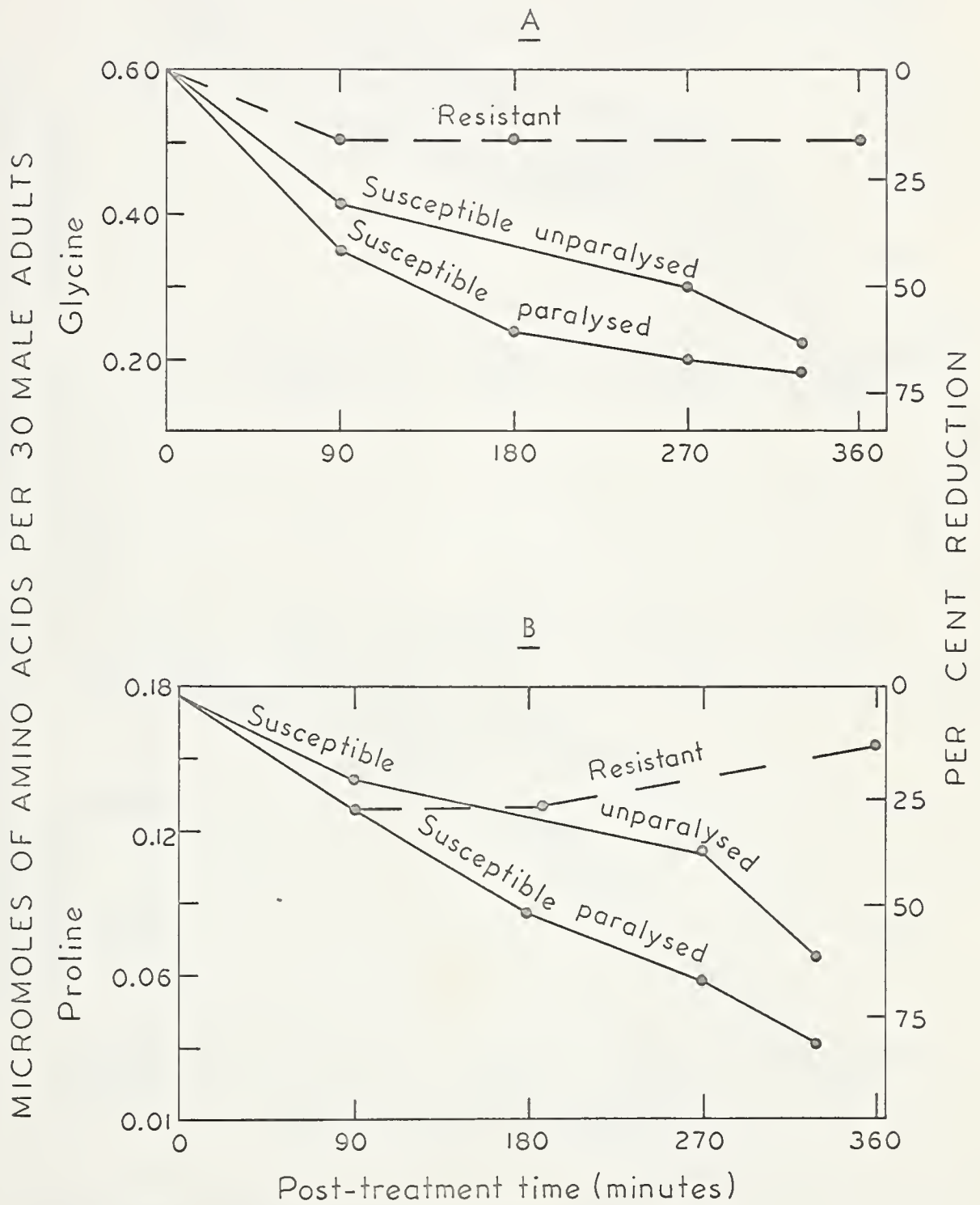
The depletion of the free amino acid pool of the blood of B. germanica, as a result of malathion toxicity was caused by the

selective effect of malathion toxicity on only four of the eighteen free amino acids present in the roach blood. The onset of toxic symptoms in the roaches appeared to be correlated with this depletion of the concentration of some of these amino acids. Slight decreases in the concentrations of only three amino acids were observed in the malathion treated resistant strain.

The selective effects of insecticidal toxicity on the free amino acids of the blood of the insects, has been reported by other workers (page 37 and 39). Joseph (1958) reported large increases in the concentration of all the amino acids of the blood of DDT poisoned meal worm. His findings were probably the result of ignoring the fact that the blood volume in the intoxicated insects is reduced. Thus he must have needed many more treated larvae than control larvae, in order to collect 0.1 ml of blood. Even in the present studies, the concentration of amino acids was found to increase by about 197 per cent in the paralysed group of roaches, per unit volume (100 ml) of blood. However, it may be recalled that the paralysed group of roaches lost about 70 per cent of the blood volume while the depletion of the amino acid pool during the same period was about 38 per cent only.

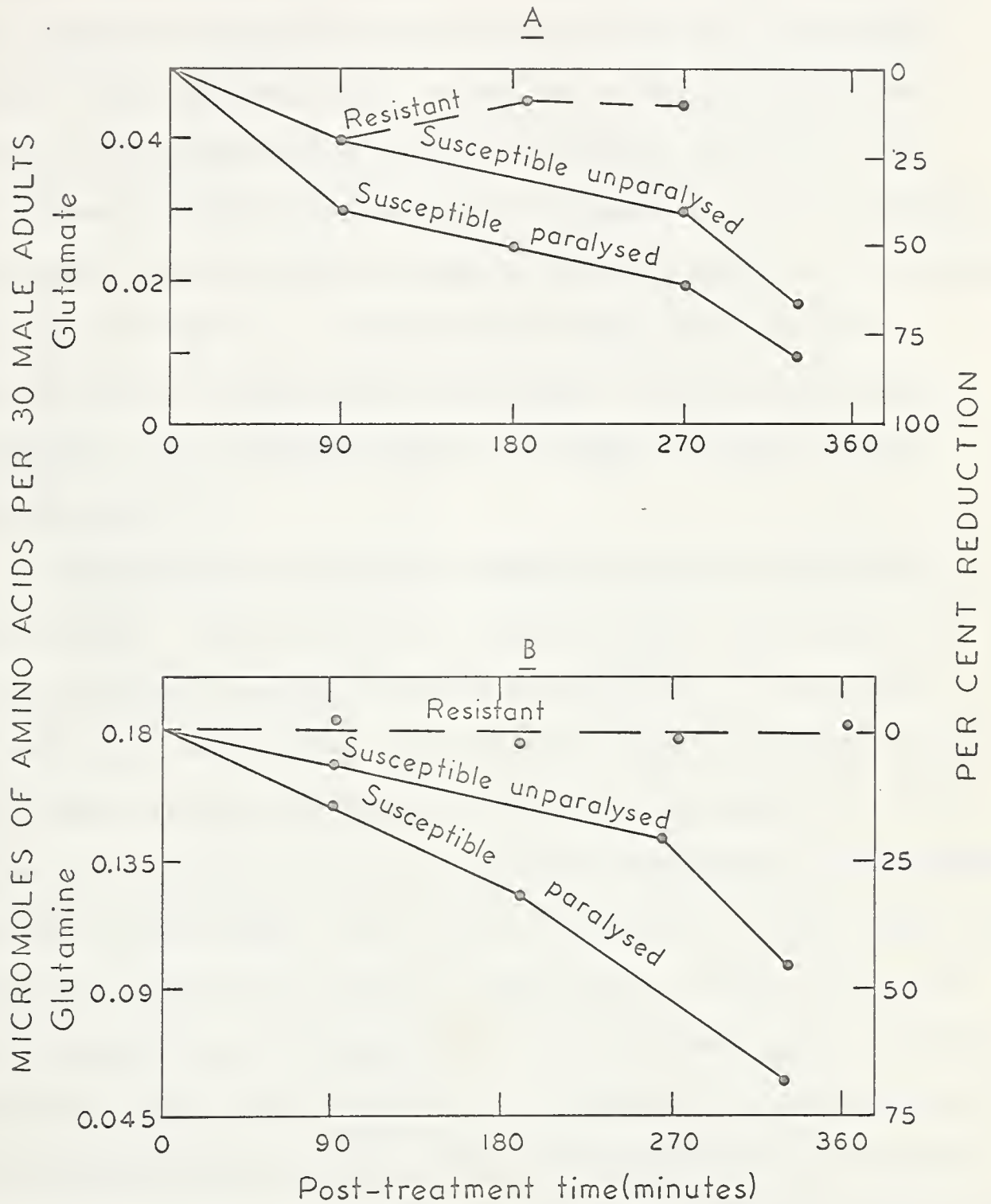
The effects of malathion toxicity were found to be selective on four amino acids only. There was not only a depletion of the concentrations of glycine, proline, glutamate and glutamine, but also great differences among the amino acids in the amount lost. This degree of selectivity on each amino acid was found to be dependent upon the degree of toxic effects on the insect. In each of the three intoxicated groups, glycine underwent the maximum reduction in its concentration, followed by proline, glutamine (except in the resistant group) and glutamate.

Figure 5: Effect of malathion* on the concentrations of free glycine and proline in the hemolymph of susceptible and resistant strains of *B. germanica*



* Dose lethal in seven hours (0.46 μ l/sq. cm.)

Figure 6: Effect of malathion* on the concentrations of free glutamate and glutamine in the hemolymph of susceptible and resistant strains of *B. germanica*



* Dose lethal in seven hours (0.46 μ l/sq. cm.)

Malathion intoxication appears to affect the concentration of the glycine more than that of other amino acids in the German roach. Even the slightest toxicity which was experienced by the resistant group, resulted in the depletion of glycine at a rate which was five times greater than that of proline and about twenty times that of glutamate (Table IV). The same selectivity, though not to the same degree, was observed in the paralysed group, which was lethally intoxicated. The higher degree of toxicity appears to have increased the rate of depletion of other amino acids more than the rate of glycine reduction. Thus glycine reduced at a rate which was only about three times more than that of proline and about ten times that of glutamate. The unparalysed group, with slightly less or delayed toxicity, had almost a similar rate of comparative depletion.

The influence of malathion toxicity on glutamine metabolism is highly selective. Malathion does not appear to affect this amino acid until the increased intoxication causes the depletion of certain other amino acids to a certain level. This selective effect was observed even in the resistant group, where glycine, proline and glutamate were found to be reduced by about 17, 11 and 10 per cent respectively of their original concentrations (Table III). With such a low depletion in these amino acids, no change was found in the glutamine concentration. However, with more pronounced toxicity in the unparalysed and the paralysed groups, the depletion of these three amino acids resulted in the reduction of glutamine also. Thus in the unparalysed group, glycine, proline and glutamate were reduced by about 63, 63, and 66 per cent respectively of their original concentrations, while glutamine was reduced by about 44 per cent. Similarly in the most

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Table III

Effect of malathion* on the reduction in the concentrations of free amino acids of the hemolymph of susceptible and resistant strains of B. germanica, in comparison with controls.

Amino acids	Percentage reduction		
	SP	SUP	R
Total	37.97	31.11	6.17
Proline	82.84	62.87	11.27
Glutamate	80.00	65.99	10.00
Glycine	70.00	63.33	16.70
Glutamine	66.67	44.45	2.78
Others	No Change		

* 5 hours of intoxication with a dose lethal in seven hours
(0.46 μ l/sq. cm.)

SP Susceptible paralysed group

SUP Susceptible unparalysed group

R Resistant strain

intoxicated group of paralysed roaches, glycine, proline and glutamate were reduced by about 70, 83 and 80 per cent respectively of their original concentration, with glutamine being reduced by about 67 per cent.

The depletion of glutamine is directly dependent upon the degree of toxicity, as judged by the amount of depletion of each of the affected amino acids and the magnitude of depletion of other amino acids, particularly proline and glutamate. Malathion toxicity appears to induce greater utilization of free amino acids, the greatest being that of glycine, followed by proline and glutamate.

The correlation between toxicity and the depletion of amino acids is further evidenced by the differences in the concentrations of each amino acid in different groups. In the paralysed group, which was most intoxicated, the depletion of proline, glutamate, glycine and glutamine were found to be about 81, 78, 64 and 68 per cent more than the depletion of these amino acids in the least intoxicated resistant strain. Even in the two groups of the susceptible strain, which differ only slightly in their reaction to malathion intoxication, the differences in the depletion of these amino acids are quite high (Table III).

There appears to be a selective correlation between the onset of toxic symptoms and the depletion of amino acids, as evidenced by the rate or amount of depletion at regular time intervals after treatment (Table II and Figures 5 and 6). Not only do the three groups of roaches differ in the quantity of amino acids reduced after five and a half hours of malathion treatment (Table II-A and II-B and III) but there is also a difference in the rate of reduction of each amino acid in each group at

different time intervals.

The curves for all the four amino acids are more steep (Figures 5 and 6) in the first ninety minutes of insecticidal action in the paralysed group, than any time after that. The values of the slope 'b' (Table IV) were found to be highest for the first ninety minutes, for almost all the amino acids. Only proline appeared to be reduced in a rather gradual fashion during the last four hours of intoxication, whereas glycine underwent about 80 per cent of its total loss in the first three hours of poisoning. Glutamate had a maximum rate of depletion during the first ninety and the last sixty minutes of intoxication. The depletion of glutamine is worth noting. Glutamine appears to be depleted similarly in the two groups of the poisoned susceptible strain up to three hours of intoxication, after which it undergoes a sharp decline in the paralysed group. The significance of this depletion of glutamine will be discussed later (page 56). In the unparalysed group, the highest value for 'b' for every amino acid except glycine, is observed to be between 270 and 330 minutes after malathion treatment. It may be recalled that this was the period when paralysis started in this group.

In the unparalysed group of roaches, the rate of depletion of glycine was maximum during the ninety minutes following treatment with malathion. The depletion of glycine may be a manifestation of hyperactivity caused by the malathion poisoning. It is also interesting to note that the paralysed group utilized almost as much of free amino acids during the ninety minutes following malathion treatment as the unparalysed group had utilized in 270 minutes after treatment.

Table IV

Value of "b" for rate of reduction of various amino acids of B. germanica for time intervals after treatment with malathion.

Amino acids	R	S	Time intervals (minutes)			
	0-90		0-90	90-180	180-270	270-330
Glycine	0.4	P	1.1	0.6	0.2	0.1
		UP	0.73	-	0.25	0.5
Proline	0.7	P	0.7	0.64	0.4	0.6
		UP	0.5	-	0.2	0.84
Glutamate	0.46	P	1.0	0.27	0.27	0.6
		UP	0.46	-	0.20	0.90
Glutamine	-	P	0.5	0.45	-	0.70
		UP	0.2	-	0.2	1.0

R Resistant strain
S Susceptible strain
P Paralysed group
UP Unparalysed group

The slight depletion of glycine, glutamate and proline in the resistant strain roaches was observed to take place only during the first ninety minutes of malathion treatment. While the glutamine concentration was almost unaffected all through the experimental period, glutamate and proline showed slight recovery after initial reduction during the ninety minutes following malathion treatment. These observations, as well as those discussed in the previous paragraphs, suggest a direct correlation between the physiological condition of the intoxicated roaches, caused by malathion poisoning and the depletion of amino acids, particularly glutamine. At this stage it is not clear if the depletion of amino acids was caused directly by malathion poisoning or is an indirect manifestation of some other biochemical lesion caused by malathion. Whatever the case may be, if ammonia accumulates the result is likely to be fatal.

Possible Metabolic Fates of Amino Acids Affected by Malathion:

The depletion of free glycine, proline, glutamate and glutamine of the blood of malathion poisoned roaches could be caused by greater rate of catabolism than anabolism, as a direct or indirect result of intoxication. These amino acids could be catabolised to any of the compounds shown in Figures 4 and 5 of the appendix.

Most of the depletion was probably the result of the utilization of amino acids by the Krebs' TCA cycle. The intoxicated roaches showed increased irritability and hyperactivity preceding knockdown, paralysis, and death. Harvey and Brown (1951) have reported increased oxygen uptake by roaches treated with a variety of organophosphates, including malathion. Similar results were obtained with German roaches treated with

malathion (Figure 3 of the appendix). The possibility of amino acids acting as a substrate for Krebs cycle is further supported by moderate inhibition of some glycolytic enzymes and depletion of α -ketoglutarate in DDT intoxicated insects (Agosin 1961 and Corrigan and Kearns 1963). Under these circumstances, the availability of substrate for amino acid synthesis may also be reduced to the extent of slowing down or even stopping the anabolic activity. Hypothetical pathways of the metabolism of four amino acids are given in Figure 8. Most of glycine was probably converted to pyruvate for final utilization by the Krebs cycle. Chefurka (1959) has reported that the major end product of carbohydrate metabolism in insects is α -glycerophosphate and that the production of pyruvate is quite slow. In an emergency, conversion of glycine to pyruvate may be expected to be a quicker way of supplying substrate for greater energy production. Thus, as in mammals, glycine would combine with N-hydroxymethyl tetrahydrofolic acid to form α -tetrahydrofolic acid-serine complex, which would then dissociate to give serine. Serine could then be deaminated to pyruvate. Enhancement of these reactions, as a direct or indirect result of intoxication is also possible.

Proline and glutamine would be expected to supply substrate to the Krebs cycle via glutamate and α -ketoglutarate. In insects free glutamate has been suggested to be in reversible equilibrium with free proline (Winteringham 1959). Kilby and Neville (1957) have indicated the participation of glutamine in transamination reactions with formation of glutamate. A decrease in the concentration of glutamate would be expected to result in depletion of proline or glutamine or both. Oxidation of glutamate by flight muscle homogenate of houseflies and sarcosome preparations has also

been reported (Sacktor and Cochran 1958). These observations suggest the apparent significance of high concentrations of free glutamine and proline in the blood of the roach, i.e. to act as substrate for Krebs cycle. In malathion intoxicated roaches, the greater need for α -ketoglutarate probably is met by its formation from proline, glutamine and glutamate, resulting in their depletion.

The depletion of glutamine in the lethally intoxicated roaches suggest that its formation from glutamate is not a significant mechanism for ammonia trapping. Corrigan and Kearns (1963) did not notice any effective role of glutamine in trapping free ammonia in DDT poisoned roaches while Winteringham (1959) indicated only temporary trapping of ammonia during abnormal conditions of DFP poisoning. Glutamine depletion was observed to follow great reductions in proline and glutamate itself (Table III). This suggests that the proline-glutamate reaction is more active than glutamine-glutamate.

Another major pathway of depletion of proline in malathion poisoned roaches is expected to be urea formation via Krebs ornithine cycle. This cycle is assumed to be present in insects. Kilby and Neville (1957) have demonstrated the presence of arginase and the production of ornithine and urea in the fat body homogenates of desert locust. McEnroe and Forgash (1957) have suggested avian-like participation of glycine in the synthesis of uric acid in insects. In the present experiments, an increase of about 40 per cent was observed in the total nitrogen of the blood of poisoned roaches. Ludwig and Bartolotta (1953) have reported an increase in urea in DDT poisoned Japanese beetles.

Fate of Ammonia and Effects of Ammonia on Roach Physiology:

The utilization of amino acids by the Krebs TCA cycle would naturally be preceded by deamination. The liberated ammonia may be excreted as such or in the form of urea, uric acid and purine metabolites (Wigglesworth 1961 and Gilmour 1961). An increase of about 40 per cent in total nitrogen and about 50 per cent in non-protein nitrogen (page 45) suggests an accumulation of nitrogenous metabolites in the blood of poisoned roaches. A 44 per cent increase in urea contents of the blood indicates that, at least, some of the free ammonia liberated in poisoned roaches forms urea, a less toxic substance than free ammonia.

The depletion of amino acids in malathion poisoned roaches is likely to reduce the efficiency of the blood in maintaining osmotic pressure. Free amino acids of the honey bee have been reported to contribute as much as 45 per cent towards the osmotic pressure of the blood (Buck 1953). The role of blood protein, which is reported to be in equilibrium with free amino acids in certain insects (Hill 1962), in maintaining the pH of the body fluid, is quite important (Buck 1953). Jochum (1956) observed the blood of parathion intoxicated silkworm larvae to become more basic. Thus changes in pH and osmotic pressure of the blood and accumulation of ammonia and nitrogenous metabolites within the body of intoxicated insects are likely to effect biochemical reactions. It is, however, hard to say at this stage as to how far the various physiological reactions are affected. A study of the effect of malathion on the physiology of excretion would be of great help in evaluating the present observations.

The Effect of Malathion on Amino Acid Metabolism in the Resistant Strain, compared with the Susceptible Strain:

The total depletion of free amino acids in the poisoned roaches of the resistant strain was only about 6 per cent of the original concentration. The susceptible paralysed group underwent about 6 times greater depletion than the resistant strain (Table III). Proline, glutamate, and glycine showed a difference of about 81, 78, and 64 per cent respectively between the two strains. Glutamine was not reduced at all in the resistant insects whereas the susceptible paralysed group registered a decrease of about 67 per cent in glutamine concentration.

It is interesting to note that in the resistant strain, the effect of malathion was manifested within the first ninety minutes after treatment, as no reduction in the concentration of any amino acid was observed after ninety minutes of intoxication. Proline and glutamate showed recovery of about 50 per cent after 90 and 180 minutes of treatment, respectively. (Table II, Figures 5 and 6.) In the susceptible strain, no such recovery was observed with any amino acid.

This recovery of amino acids may be compared with the in vivo recovery of cholinesterase in an organophosphate-intoxicated resistant strain of houseflies (Mengle and Casida 1960) and in B. germanica, (Figure 2 of the appendix). The German roaches were found to have about 30 per cent inhibition in the in vivo activity of cholinesterase of the brain, in the first two hours after insecticidal treatment. (The insects were treated with the concentration of malathion which was lethal to the susceptible strain within 7 to 8 hours.) A recovery in the activity of cholinesterase was recorded after three hours of poisoning (Figure 2 of the appendix). These observations indicate a close relationship

between the inhibition and subsequent recovery of cholinesterase and the physiological condition that brings about depletion and subsequent recovery of glutamate and proline in the resistant roaches.

4. Conclusion:

Malathion intoxication caused depletion of the total free amino acid concentration of the blood of B. germanica. Of the 18 free amino acids present in the blood, 14 did not reflect any change with malathion intoxication. A direct positive correlation between the degree of intoxication and the amount of depletion of glycine, proline, glutamate and glutamine was found. These amino acids (except for glutamate) are those present in the highest concentrations in the blood. Differences in the rates of reduction of these amino acids also depend upon the degree of intoxication. The depletion of amino acids was correlated with the onset of toxic symptoms. Glutamine was reduced only after acute toxicity caused considerable reduction in the concentrations of the three amino acids.

Most of the depletion of amino acids was caused by their utilization by the Krebs cycle to meet greater energy requirements of the poisoned roaches. Most of the glycine could have been converted to pyruvate. Proline, glutamine and glutamate could have converted to α -ketoglutarate.

The depletion of glutamine suggests a complete break down of all glutamine-asparagine mechanism of ammonia transportation. An increase in urea contents of the blood of poisoned roaches suggests that the ornithine cycle was involved in the trapping of ammonia liberated by deamination of amino acids. The non-protein and total nitrogens also increased in the

blood of poisoned roaches. The accumulation of nitrogenous metabolites is likely to affect the efficiency of the blood in maintaining pH and osmotic pressure, besides being toxic in itself.

V THE EFFECT OF MALATHION ON THE METABOLISM OF LABELLED
GLUCOSE, GLUTAMATE AND GLYCINE IN SUSCEPTIBLE AND
RESISTANT STRAINS OF B. GERMANICA.

1. Introduction:

The enhanced catabolism of free glycine, proline, glutamate and glutamine in the hemolymph of malathion intoxicated roaches and the possible utilisation of these amino acids by the Krebs' cycle was discussed in the previous chapter. To confirm this circumstantial evidence the metabolism of uniformly labelled glucose, glutamate and glycine was studied in poisoned and unpoisoned roaches. These studies were performed on resistant and susceptible strains of German roaches to obtain additional information on the relationship between resistance to malathion and metabolism of free amino acids.

2. Materials and Methods:

2.1 Materials:

The materials used and the sources of supply are listed on page 107 of the appendix.

2.2 Methods:

2.21 Administration of Labelled Compounds:

Glucose (U) C^{14} , glutamate (U) C^{14} and glycine (U) C^{14} were administered to three groups of roaches (malathion poisoned roaches of the susceptible and the resistant strain and to the unpoisoned susceptible strain controls) by hyperdermic injections. The labelled compounds were dissolved in distilled

water. The quantities of the compounds injected per insect were as follows: about 0.0015 micromoles of glucose (U) C^{14} with a radioactivity of about 3000 counts per minute, 0.0017 micromoles of glutamate (U) C^{14} with a radioactivity of about 2500 counts per minute and 0.007 micromoles of glycine (U) C^{14} with a radioactivity of about 6000 counts per minute.

Adult males of the susceptible and the resistant strains were treated with malathion in the usual way (page 7). Injections were performed immediately after malathion treatment. The hyperdermic needle of a micrometer syringe was introduced at the intersegmental membrane of the first and second abdominal segment, just below the pleuroventral line, and 0.003 ml of the solution was injected. Individuals that bled after injection were rejected.

2.22 Collection of Carbon Dioxide:

After the injections of the radioactive compounds, the roaches of each replicate of each treatment (three replicates) were kept separately, each in a 100 ml Warburg type flask with no side arms. The centre well which contained two ml of saturated potassium hydroxide solution, was covered with a wire netting cap. After the introduction of the roaches, the flasks were immediately closed with serum rubber caps and placed in a constant water bath at 30°C. From time to time the flasks were gently shaken to facilitate the absorption of carbon dioxide. After every hour, two ml of fresh potassium hydroxide was introduced into the centre well after completely removing the already present potassium hydroxide with absorbed carbon dioxide. This was done by inserting a long hyperdermic needle through the rubber cap. The potassium hydroxide, which was removed

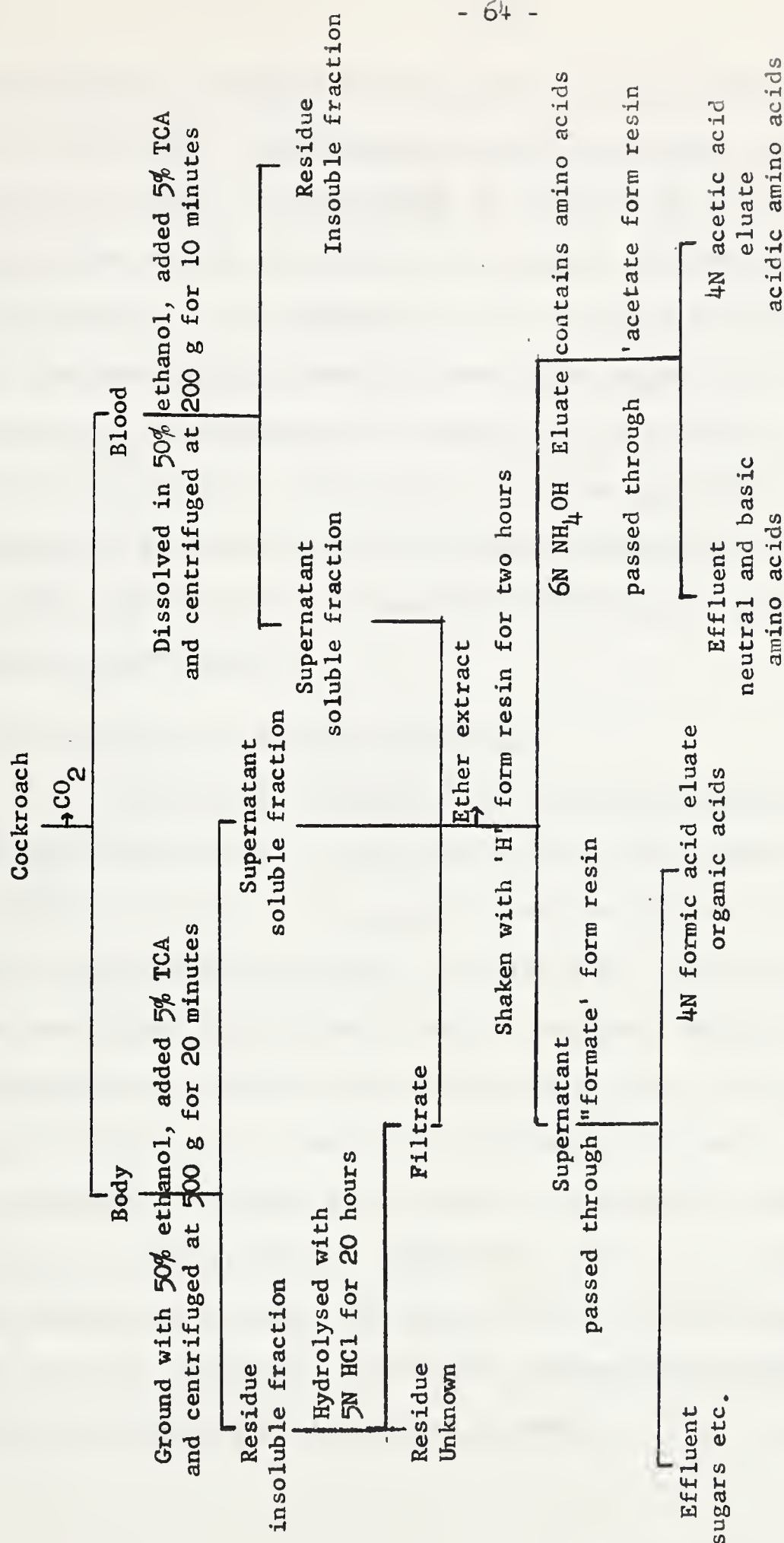
from the flask, was transferred to a tube containing 3 ml of saturated barium hydroxide solution. One per cent of sodium carbonate solution was then added to give 10 - 20 mg of barium carbonate precipitate. The solution was filtered through a weighed filter paper. After thorough drying at 85°C for three hours, the precipitates were weighed and the radioactivity assayed using a Gieger-Muller tube with a Mica end window (density 1.4-2 mg/sq. cm.) with about 2 per cent efficiency all counts were corrected for background and self-adsorption.

2.23 Separation of Soluble and Insoluble Fractions of Blood and Body:

The treated insects were killed in ether and the blood was collected in the usual way. The flow sheet on the next page gives the different steps of collecting various fractions. The soluble fraction was collected by dissolving blood in 50 per cent ethanol and adding 2 ml of 5 per cent trichloroacetic acid, shaking the tube thoroughly and centrifuging the tube at 200 g for ten minutes. The supernatant was decanted and the residue was washed three times, each time with 2 ml of trichloroacetic acid followed by five minutes of centrifugation and then decantation. The supernatants of each washing were pooled together and dried. This gave the soluble fraction of blood. The residual precipitate obtained by the action of trichloroacetic acid on the blood, formed the insoluble fraction of the blood and contained protein and glycogen etc. The dried soluble fraction was further washed three times with 10 ml of diethyl ether. The ether extract contained lipids.

The soluble and insoluble fractions of the body were obtained by homogenizing the bodies of roaches after blood removal. Homogenates

Scheme for separation of various fractions and compounds from the body of *B. germanica*.



were prepared by grinding the bodies with 30 ml of cold 50 per cent ethanol in a hand blender. The homogenates were centrifuged at 500 g for twenty minutes at -10°C . The supernatant was decanted and the residue washed three times with 15 ml of 50 per cent ethanol, followed by centrifugation and decantation. The residue formed the insoluble fraction of the body. The supernatant and the washings were pooled together and the colloidal protein was precipitated out by adding 20 ml of cold 5 per cent trichloroacetic acid. The protein precipitate was added to the insoluble fraction obtained above, while the soluble supernatant was dried in vacuo at 40°C . The dried fraction was further washed with 30 ml of diethyl ether to remove lipids.

2.24 Hydrolysis of Insoluble Fractions:

The insoluble fraction of the blood containing mainly protein was hydrolysed with 3 ml of 5N hydrochloric acid in vacuum sealed tubes at 100°C for 20 hours. The insoluble body fraction was hydrolysed with 60 ml of 5N hydrochloric acid at low heat under reflux for 20 hours. The hydrolysate was filtered in vacuum. Material resistant to acid hydrolysis was filtered out onto filter paper disks, washed thoroughly with distilled water, dried and its radioactivity counted. This material is designated as unknown in the tables V, VIII and XI. The filtrate was dried on a flash evaporator, redissolved in water and dried again. This was repeated three times. The last traces of acid were removed by passing air for about 30 minutes. Lipids were removed from the dried fraction by washing with 30 ml of diethyl ether as usual.

2.25 Separation of Amino Acids, Sugars and Organic Acids etc:

The same procedure was followed for soluble and hydrolysed insoluble fractions. Amino acids were separated from the rest of the compounds by allowing the 'H' form resins to adsorb amino acids. The dried fractions were redissolved in 5 ml of water and shaken in rotary shaker for two hours with 500 mg of Nalcite HCR 'H' form resin (Fraenkel-Convat et al. 1955). (The resins were obtained in hydrogen form.) This weight of resin was found to be at least five times in excess of the quantity sufficient to ensure complete adsorption of amino acids. The supernatant that contained sugars, sugar phosphates and organic acids was removed by a pipette. The resin was washed five times, each time by shaking with 5 ml of water. The supernatant and the resin washings were pooled together to get the fraction containing sugars and acids etc. The amino acids were eluted from the resin by shaking with 5 ml of 5 N ammonium hydroxide three times.

2.26 Separation of Acidic Amino Acids from Neutral and Basic Amino Acids:

The amino acid eluate obtained from the previous procedure was dried and traces of acids removed as described before. The dried and almost neutral eluates of each replicate were pooled together, redissolved in 5 ml of water and shaken in a rotary shaker for two hours in the presence of 500 mg of AG 1 x 4 resin in acetate form. (Acetate form resin was generated from chloride form of Dowex 1 x 10 resin according to the method of Canvin and Beevers (1961). 50 ml of 1 M sodium acetate was passed through a resin column of 6 x 1 cm until silver nitrate gave negative chloride

test. 50 ml of 0.1 N acetic acid was now passed through the column followed by distilled water until the pH of the column was between 5.8 and 7.) The supernatant was removed and the resin washed five times with water as described above. The supernatants and the washings of the resin contained the neutral and basic amino acids. The glutamate and aspartate remained adsorbed on the resin. These were eluted from the resin by shaking with 5N acetic acid and repeating the process three times.

After the addition of 150 ml of 2 N hydrochloric acid, the neutral and basic amino acid fraction was dried in vacuo at 40°C. This process resulted in the hydrolysis of glutamine and asparagine to glutamate and aspartate respectively. The hydrolysate was made acid-free by repeated drying in vacuo. Glutamate and aspartate were removed from this hydrolysate by shaking with acetate form resin as described above. Thus the amino acids were obtained in three mixtures, the first containing glutamate and aspartate, the second containing hydrolysed glutamine and asparagine and the third containing neutral and basic amino acids.

2.27 Separation of Sugars and Organic Acids:

The supernatant of the Nalcite HCR 'H' form resin treatment containing sugars and organic acids and phosphates was passed through a 1 x 6 cm column of Dowex 1 x 10 resin in formate form. (The formate form of resin was generated from chloride form by using 100 ml of 1 M sodium formate and 0.1 N formic acid and following the procedure of Canvin and Beevers (1961) for the generation of acetate form resin.) Neutral sugars passed through the column which was washed with 60 ml of water. The

organic acids were eluted from the column with 40 ml of 4 N formic acid.

2.28 Separation and Identification of Individual Compounds:

Radioactive compounds of sugars and amino acid fractions were separated and identified by descending paper chromatography and high voltage electrophoresis. Samples of each replicates were pooled for this purpose.

Sugars were separated by descending paper chromatography in n-propanol, ethyl acetate and water (7 : 1 : 2, v/v/v) for 20 - 24 hours. Serrated Whatman No. 1 filter paper was used. The spots of glucose and disaccharide were located by autoradiography. The Rf value of disaccharide trehalose were confirmed from the relative Rf values of compounds given by Treherne (1960).

Separation of glutamate and aspartate was achieved by using one dimensional descending paper chromatography with phenol and water (4 : 1, v/v) as the solvent in an ammonia (0.3 per cent) saturated chamber (Block 1958).

The neutral and basic amino acids were separated by two dimensional paper chromatography in phenol-water and butanol - butyl acetate solvent systems (Page 21). Amino acids that incorporated labelled carbon glucose (U) C^{14} were satisfactorily separated by this method, whereas high-voltage electrophoresis was found to be unsuitable for the detection of small quantities of radioactivity. After the assay of radioactivity, the spots were eluted and chromatographed in phenol pH 12 solvent (page 21). Spots were developed with 0.1 per cent ninhydrin in acetone and the Rf values compared with the known amino acids. A similar procedure of separation was followed for glutamate (U) C^{14} experiments. The presence of large amounts

of unutilized glycine in the samples obtained from glycine (U) C^{14} experiments resulted in streaking in the positions of serine, cystine, glutathione, glucine, alanine and proline. In these cases, good separation of amino acids was achieved by one dimensional electrophoresis after the method of Dryer and Bymun (Personal communication to Dr. L. B. Smiller). The electrophoresis apparatus was supplied by Gilson Medical Electronics, Middleton, Wisconsin. Spots were applied at one end (anode) of a 4 ft x 1 ft Whatman No. 3 paper, which was then wet with 8 per cent formic acid, pH 1.8 and subjected to electrophoresis at 4000 volts and 200 milliamperes, for 90 minutes (Procedure Manual - High voltage electrophoresis tank - by Servonuclear Corporation, 28-21 Astoria Boulevard, Long Island City, New York, U.S.A.). Good separation of glycine, alanine, serine, proline and glutathione was achieved (Figure 7). Spots of known amino acids were developed by dipping the paper in ninhydrin solution (Atfield and Morris 1961). After the assay of activity, the spots were eluted and chromatographed in butanol-butyl acetate solvent (page 21) along with the known compounds as markers.

2.29 Autoradiography and Assay of Activity:

The radiochromatograms were exposed to Kodak "No screen" X ray films for about four weeks. The films were developed according to manufacturers instructions. The radioactive areas revealed by autoradiography were eluted with 30 per cent ethanol and the solution counted on metal planchets in a Geiger-Muller tube with a Mica end window. A portion of each fraction of every step was also read for radioactivity in a similar way.

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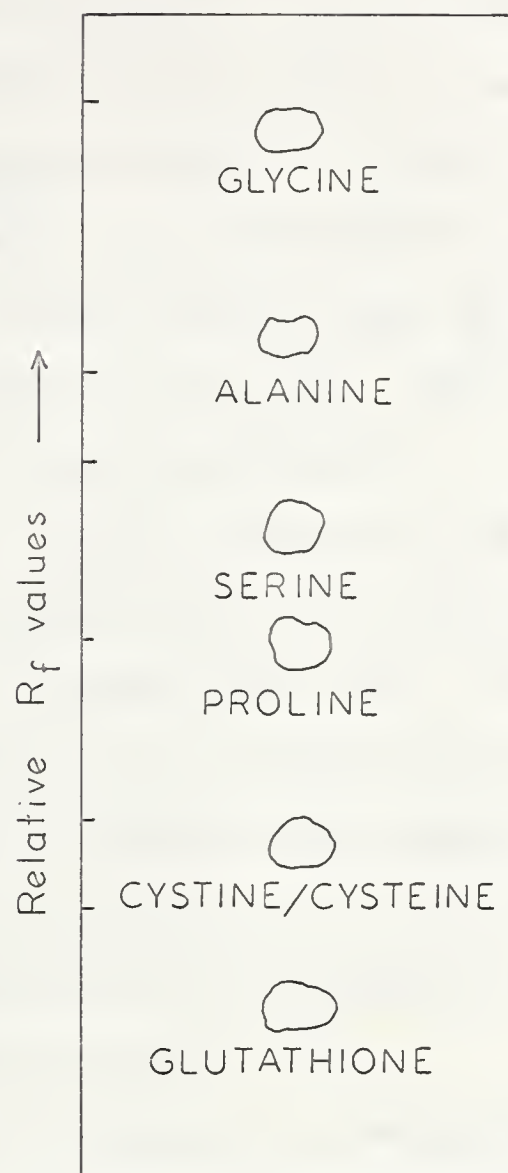
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Figure 7. Separation of amino acids by high voltage electrophoresis.



pH 2.2 at 4000 volts, 200 milliamperes for 90 minutes.

3 Results and Discussion:

3.1 Results:

The effects of malathion toxicity on the metabolism of glucose (U) C^{14} , glutamate (U) C^{14} and glycine (U) C^{14} in the resistant and susceptible strains of B. germanica are presented in Tables V to XIII. Labelled carbon from these compounds was incorporated into carbon dioxide, organic acids and phosphates, neutral compounds which mainly contain sugars, into glycogen, lipids, protein and amino acids. Some radioactivity was also found in excreted and regurgitated material.

Besides the compounds presented in the Tables V to XIII, there may have been other compounds which did not incorporate enough labelled carbon to be detected by the GM counter used. It may be noticed that the recovery of radioactivity is not the same in all the experiments. Moreover, the radioactivity of the individual amino acids did not add up to the total activity recorded before spotting on the filter paper. This was probably due to loss of radioactivity by self adsorption as well as in the compounds which had incorporated low amounts of C^{14} .

The figures presented in the Tables IV to XIII represent the incorporation of C^{14} of glucose (U) C^{14} , glutamate (U) C^{14} and glycine (U) C^{14} , into various compounds in the five hours experimental period, based upon the amount of each compound injected. The rates of uptake and metabolism of each compound may not be the same. Thus a correction factor will have to be applied for each compound in order to compare the results on the basis of the amounts of compound actually catabolised. For instance 33 per cent of the injected glycine (U) C^{14} was recovered as such from the

control roaches (Table XI), while 67 per cent of it was catabolised. About 13 per cent of the injected labelled glycine was released as $C^{14}O_2$. This 13 per cent is actually 19.5 per cent of the 67 per cent catabolised glycine $(U)C^{14}$. Similarly 27.6 per cent of the injected glycine $(U)C^{14}$ that was incorporated into other amino acids is equal to 41.4 per cent of the catabolised glycine. Thus, to base the figures on the amount of each injected compound catabolised and not on the amount of each compound injected, the figures in the glucose $(U)C^{14}$ experiments will have to be multiplied by 1.04, those of the glutamate $(U)C^{14}$ experiments by 1.17 and that of glycine $(U)C^{14}$ experiments by 1.5. However, these figures would not affect the comparative values within each experiments.

3.2 Discussion:

Metabolism of the labelled compounds:

About one third of the injected glucose $(U)C^{14}$ was released as $C^{14}O_2$ while about one tenth was converted into organic acids (Table V). These observations are consistent with the operation of the Embden-Meyerhof sequence of glycolysis and of the Krebs' cycle. The alternate pathway of the pentose cycle was found to have been responsible for only 4 - 9 per cent of glucose metabolism in American roaches (Silva et al. 1958).

About one fourth of the labelled carbon of the injected glucose $(U)C^{14}$ was found to have been incorporated into carbohydrates: 14 per cent as trehalose and 11 per cent as glycogen (Table V) and Treherne (1960) has reported trehalose to be the major blood sugar in insects. A trehalose splitting enzyme has been purified by Kalf and Reider (1958). Treherne (1960) has reported the presence of more trehalose than glucose in the

Table V

The effect of malathion on the incorporation* of glucose (U)¹⁴C, into various compounds in resistant and susceptible strains of B. germanica.

Compounds	Control		Resistant ¹		Susceptible ¹	
	Activity ²	Percent	Activity	Percent	Activity	Percent
Carbon dioxide	17184	34.37	19230	38.46	29768	59.54
Amino acids	4591	9.18	3954	8.70	2508	5.01
Glucose	1907	3.81	1437	2.87	787	1.57
Trehalose	6910	13.82	5863	11.72	1718	3.44
Glycogen	5528	11.06	4864	9.73	1760	3.52
Organic acids	5160	10.32	5400	10.80	6055	12.11
Lipids	370	0.70	378	0.78	360	0.72
Protein	1150	2.30	1100	2.00	670	1.30
Excretion	840	1.68	680	1.36	1000	2.00
Unknown	400	0.40	400	0.40	450	0.45
Total	44040	87.64	43306	87.02	45076	89.66

* Five hours of metabolism
1 Five hours of malathion intoxication
with a dose lethal in seven hours
(0.46 µl/sq. cm.)
2 Radioactivity in counts per minute
3 Percent of injected radioactivity

Table VI

The effect of malathion on the incorporation* of glucose (U)C¹⁴, into various compounds in resistant and susceptible strains of B. germanica.

Compounds	Control Activity ²	Resistant ¹		Susceptible ¹	
		Activity	% Difference from control	Activity	% Difference from control
BLOOD					
Protein	65	60	-7.69	48	-26.15
Glucose	197	110	-44.17	97	-50.79
Trehalose	1000	850	-15.00	518	-48.20
Organic acids	460	500	+8.70	485	+45.40
BODY					
Protein	1085	1040	-4.15	522	-51.89
Glucose	1710	1327	-22.39	690	-59.65
Trehalose	5910	5013	-15.18	1200	-79.69
Glycogen	5528	4864	-12.01	1760	-68.17
Organic acids	4700	4900	+4.26	5570	+18.51
Lipids	370	378	+2.16	110	-70.29

* Five hours of metabolism
1 Five hours of malathion intoxication with a dose lethal in seven hours (0.46 μ /sq. cm.)
2 Radiocativity in counts per minute

Table VII

The effect of malathion on the incorporation* of glucose (U)¹⁴C, into free amino acids of resistant and susceptible strains of B. germanica.

Amino acids	Control		Resistant ¹		Susceptible ¹	
	Activity ²	Activity	Activity	Difference from control	Activity	Difference from control
BLOOD						
Aspartate	48	52		+8.33	46	-4.17
Glutamate	177	188		+6.21	142	-19.8
Glutamine- asparagine	200	195		-2.5	130	-35.0
Others	808	654		-19.06	345	-57.30
BODY						
Aspartate	250	240		-4.00	255	+2.00
Glutamate	554	387		-30.15	248	-55.23
Glutamine- asparagine	500	560		+12.00	365	-27.00
Serine	200	1590			175	-12.5
Glycine	785				357	-54.52
Alanine	203			-11.8	200	-1.5
Proline	600				245	-59.17

Legend as in Table VI

nerve cord of American roaches. Winteringham (1959) has reported conversion of about 60 per cent of glucose C^{14} into trehalose and glycogen in five hours by houseflies.

Very little labelled carbon of glucose $(U)C^{14}$ was incorporated into protein (2.3 per cent of the injected amount). Hydrolysis of the protein showed both the acidic and basic and neutral amino acids had incorporated labelled carbon. Winteringham and Harrison (1956) found that almost all the incorporation of acetate $(2)C^{14}$ into protein was due to aspartate and glutamate of the thoracic protein of houseflies. They further suggested that these two protein amino acids are in equilibrium with the free amino acids of the blood and body.

Free amino acids incorporated only about ten per cent of the labelled carbon of glucose $(U)C^{14}$ (Table V). Aspartate, glutamine-asparagine, serine and glycine, glutamate, alanine and proline were found to contain C^{14} (Table VII). In the blood, the ratio between the acidic amino acids and the rest of the amino acids that had incorporated radioactivity was found to be 1 : 6.5, where as in the body, this ratio was increased to 1 : 2. The low ratio of the blood acidic and other amino acids is consistent with the presence of small quantities of free acidic amino acids in the blood (Table I). Except for serine, alanine and aspartate, the other four amino acids were observed to have incorporated fairly high amounts of C^{14} . The incorporation into alanine could be explained by the glycolytic breakdown of glucose to pyruvate. Pyruvate entering Krebs' cycle would be converted to α -ketoglutarate and then to oxalacetate. Alanine aspartate and glutamate might then be formed by transamination reaction as follows:-

glutamate + pyruvate ----- α -ketoglutarate + alanine
 α -ketoglutarate + aspartate ----- glutamate + oxalacetate .

The incorporation of high amounts of labelled carbon into glutamine, asparagine, proline and glycine is consistent with the presence of high amounts of these amino acids in the blood of the German roaches (Table I). Glutamate, which is normally present in low concentrations in German roach blood, was found to have incorporated rather high amounts of labelled carbon (Table VII). After a few more hours, it is likely that more labelled carbon of this glutamate would have been incorporated into proline. Conversion of glutamate into proline and glutamine had been discussed on page 36. The presence of radioactive serine suggests the possible synthesis of glycine from 3-phosphoglycerate via serine, as discussed on page 32.

It is interesting to note that in five hours, as much as 31 per cent of the metabolised glutamate $(U)C^{14}$ (26 per cent of the injected amount, Table VIII), was released as $C^{14}O_2$, as compared to about 35 per cent of $C^{14}O_2$ obtained after glucose $(U)C^{14}$ catabolism. This suggests a rapid conversion of glutamate to α -ketoglutarate which is then decarboxylated by the reactions of Krebs' cycle.

Neutral compounds, mainly sugars and glycogen, had incorporated about 9 per cent of the labelled carbon of the injected glutamate $(U)C^{14}$ (Table VIII). Glucose synthesis can be achieved via Krebs' cycle from α -ketoglutarate and reversal of Embden-Myerhof sequence of glycolytic reactions. Thus, like mammals, insects can also be expected to utilize free amino acids for gluconeogenesis. Once the amino acids have taken part in the synthesis of pyruvate, they can also participate in the formation of lipids. However, Tables VIII and XI suggest that this reaction is not very efficient in this insect.

Table VIII

The effect of malathion on the incorporation* of glutamate (U)¹⁴C, into various compounds in resistant and susceptible strains of B. germanica.

Compounds	Control		Resistant ¹		Susceptible ¹	
	Activity ²	Percent ³	Activity	Percent	Activity	Percent
Carbon dioxide	18504	26.43	20643	29.49	33559	47.94
Amino acids	25737	36.77	25912	37.02	15652	22.36
Sugar	5515	7.88	3580	5.11	1830	2.62
Glycogen	1050	1.50	960	1.37	800	1.15
Organic Acids	4600	6.57	5410	7.73	6625	9.46
Protein	3750	5.36	3594	5.13	3465	4.95
Lipids	460	0.66	540	0.77	485	0.69
Excretion	1833	2.62	1950	2.79	1790	2.55
Unknown	460	0.66	530	0.76	450	0.64
Total	61909	88.45	63119	90.17	64656	92.36

* Five hours of metabolism
 1 Five hours of malathion intoxication with a dose lethal in seven hours (0.46 µl/sq. cm.)
 2 Radioactivity in counts per minute
 3 Percent of injected radioactivity

Table IX

The effect of malathion on the incorporation* of glutamate (U)¹⁴C, into various compounds in resistant and susceptible strains of B. germanica.

Compounds	Control Activity ²	Resistant ¹		Susceptible ¹	
		Activity	% Difference from control	Activity	% Difference from control
BLOOD					
Protein	665	680	+2.26	650	-2.26
Sugars	790	560	-16.46	290	-63.29
Organic acids	657	752	+14.45	1270	+93.30
BODY					
Protein	3085	2915	-5.51	2815	-8.75
Sugars	4725	3080	-34.81	1540	-67.41
Glycogen	1050	960	-8.57	800	-23.80
Organic acids	3943	4658	+18.11	5355	+36.57
Lipids	460	540	+17.39	485	+5.43

* Five hours of metabolism
1 Five hours of malathion intoxication with a dose lethal in seven hours (0.46 µl/sq. cm.)
2 Radiocativity in counts per minute

Table X

The effect of malathion on the incorporation* of glutamate (U)¹⁴C into free amino acids of resistant and susceptible strains of B. germanica.

Amino acids	Control		Resistant ¹		Susceptible ¹	
	Activity ²	Activity	% Difference from control	Activity	% Difference from control	
BLOOD						
Aspartate	287	320	+11.50	315	+9.76	
Glutamate	1357	1434	+5.67	1260	-7.15	
Glutamine-asparagine	315	355	+12.70	340	+7.93	
Others	770	560	-27.27	250	-64.94	
BODY						
Aspartate	1387	1320	-4.83	1350	-2.67	
Glutamate	7654	7450	-2.67	6014	-21.43	
Glutamine-asparagine	1040	1195	+14.42	1260	+21.15	
Serine	570	630	+10.53	520	-8.77	
Proline	7127	6822	-4.28	1350	-81.06	
Alanine	610	590	-3.27	570	-6.56	
Glycine	2870	3020	+5.22	1560	-45.64	
Arginine	340	300	-11.76	-	-	

Legend as for Table IX

Both the acidic and basic and neutral amino acids of the body protein were observed to have incorporated about 5 per cent of the injected glutamate (U) C^{14} . Similar observations were found with the glucose (U) C^{14} experiments.

Seven free amino acids were found to have incorporated C^{14} from glutamate (U) C^{14} (Table X). Aspartate and alanine could have been formed by the transamination reaction discussed earlier. Both glutamate and aspartate can combine with ammonia and form glutamine and asparagine respectively. Proline and glutamate are reported to be in reversible equilibrium in insects (page 55). Incorporation of labelled carbon into arginine (Table X) suggests transamination of glutamic semialdehyde, to form ornithine, which by picking up CO_2 and two molecules of ammonia can form arginine via citrulline. This indicated the role of free amino acids in ammonia excretion in insects and has been discussed on page 56 in connection with the observed increase of non-protein and total nitrogens and the urea contents of the blood of malathion treated roaches.

Labelled carbon of glutamate (U) C^{14} was also incorporated into serine and glycine, probably through 3-phosphoglycerate. The glycine mechanism appears to be very efficient in roaches.

The metabolism of glycine through the Krebs' cycle appears to be slower than that of glucose and glutamate. Only about 20 per cent of the metabolised glycine (U) C^{14} (13 per cent of the injected amount) was released as $C^{14}O_2$ (Table XI). Considering the comparatively low incorporation of labelled carbon of glycine (U) C^{14} into $C^{14}O_2$, its incorporation into organic acid fraction seems to be fairly high (Table XI). This may be due

to the formation of glyoxylate or hippuric acid. The production of these compounds has been shown to occur in insects (Fukuda and Hayashi 1958, Friedler and Smith 1954).

Free glycine of the insect body is also used for the production of carbohydrate, a process observed with glutamate $(U)C^{14}$ experiments also. Most of the 10 per cent incorporation of labelled carbon of the metabolised glycine $(U)C^{14}$ (7 per cent of injected amount) must have taken place by the reversal of the anabolic reactions which is probably via 3-phosphoglycerate. As in glutamate $(U)C^{14}$ experiments, the glycine $(U)C^{14}$ experiments also show about 6 per cent of the metabolised glycine $(U)C^{14}$ incorporated into body protein. A comparison of the protein, lipid and carbohydrate synthesis from glutamate and glycine (Tables VIII and XI) suggests greater preference for storage of reserve food in the form of carbohydrate rather than protein or lipids.

Free amino acids were observed to have incorporated about 28 per cent of the labelled carbon of injected glycine $(U)C^{14}$ (41 per cent of metabolised amount). Glutamate, glutamine-asparagine, serine, glutathione, aspartate, alanine and cystine/cysteine showed maximum incorporation of labelled carbon (Table VIII). A similar pattern was observed in the free amino acids of the blood and body. Serine had probably been formed as an intermediate compound during the metabolism of glycine to pyruvate, according to the sequence of reactions discussed on page 55. Both glutamate and aspartate could then be synthesized by the transamination of α -ketoglutarate and oxalacetate respectively, obtained by the metabolism of pyruvate via Krebs cycle. However, direct transamination of α -ketoglutarate by ammonia donated by glycine has been demonstrated in the silk worm by Fukuda and

Table XI

The effect of malathion on the incorporation* of glycine (U)¹⁴C, by various compounds into resistant and susceptible strains of B. germanica.

Compounds	Control		Resistant ¹		Susceptible ¹	
	Activity ²	Percent ³	Activity	Percent	Activity	Percent
Carbon dioxide	13180	13.18	14300	14.30	18870	18.87
Amino acids	27630	27.60	27000	27.00	21212	21.20
Glycine	32560	32.56	30000	30.00	25470	25.47
Sugars	4890	4.90	4900	4.90	3600	3.60
Glycogen	2100	2.10	2216	2.20	2000	2.00
Organic acids	6240	6.24	6740	6.74	9340	9.34
Protein	3900	3.90	3500	3.50	3800	3.80
Lipids	514	0.50	500	0.50	340	0.34
Excretion	1460	1.50	1800	1.80	2700	2.70
Unknown	400	0.40	400	0.40	260	0.26
Total	92874	92.87	91356	91.36	87592	87.59

* Five hours of metabolism
 1 Five hours of malathion intoxication with a dose lethal in seven hours (0.46 µl/sq. cm.)
 2 Radioactivity in counts per minute
 3 Percent of injected radioactivity

Table XII

The effect of malathion on the incorporation* of glycine (U)¹⁴C, into various compounds in resistant and susceptible strains of B. germanica.

Compounds	Resistance ¹		Susceptible ¹	
	Control Activity ²	Activity % Difference from control	Activity % Difference from control	% Difference from control
BLOOD				
Protein	740	650 -12.16	840	+13.5
Sugars	670	505 -24.62	290	-56.71
Organic acids	975	910 -6.67	1065	+9.23
BODY				
Protein	3160	2850 -9.81	2960	-6.33
Sugars	4220	4395 +3.93	3510	+16.82
Glycogen	2100	2216 +4.1	2000	-4.1
Organic acids	5064	5830 +15.12	8275	+63.4
Lipids	514	500 -2.7	340	-32.00

* Five hours of metabolism
1 Five hours of malathion intoxication with a dose lethal in seven hours (0.46 µl/sq. cm.)
2 Radioactivity in counts per minute

Table XIII-A

The effect of malathion on the incorporation* of glycine (U)¹⁴C into free amino acids of the blood of resistant and susceptible strains of B. germanica.

Amino acids	Control		Resistant ¹		Susceptible ¹	
	Activity ²	Activity	Activity	% Difference from control	Activity	% Difference from control
Unutilised glycine	3115	2800		-10.11	2120	-31.94
Glutamate-aspartate	710	678		-4.51	800	-12.67
Serine-proline	438	480		+9.58	378	-13.69
Glutamine-asparagine	498	580		+16.67	625	+25.50
Glutathione	235	210		-10.64	250	+6.38
Cystine/cysteine	125	140		+11.11	110	-11.11
Alanine	218	240		+14.68	220	+0.09

* Five hours of metabolism

1 Five hours of malathion intoxication with a dose lethal in seven hours (0.46 µl/sq. cm.)

2 Radioactivity in counts per minute

Table XIII-B

The effect of malathion on the incorporation* of glycine (U)¹⁴C into free amino acids of the body of resistant and susceptible strains of B. germanica.

Amino acids	Resistant ¹		Susceptible ¹	
	Control Activity ²	Activity % Difference from control	Activity	% Difference from control
Unutilized glycine	29445	27200	23350	-20.69
Glutamate	4680	4015	2050	-56.19
Aspartate	2438	2585	2460	+0.9
Glutamine-asparagine	2948	3090	3698	+25.79
Serine-proline	2684	2495	2465	-8.16
Alanine	2105	2260	2160	+3.09
Glutathione	2500	2460	2585	+4.00
Cystine/cysteine	1868	1800	1740	-6.85

Legend as for Table XIII-A

Hayashi (Gilmour 1961) and Kilby and Neville (1957) in locust fat body. Glutamine and asparagine formation could have taken place in the normal way. The synthesis of proline from glycine must have been via glutamate. Alanine could have been formed from pyruvate. Bricteux-Gregoire et al. (1958) have demonstrated the incorporation of glycine into alanine in silkworms.

Glycine (U) C^{14} has been found to enter into the synthesis of the tripeptide glutathione (Tables XIII A and B). Glutamate and cysteine form the dipeptide glutamylcysteine, which combines with glycine to form glutathione. About 0.19 per cent of labelled carbon of glycine (U) C^{14} was incorporated in cystine/cysteine. Insects, therefore, appear to follow the mammalian pathway of cysteine synthesis from glycine via serine and cystathione. In mammals cysteine and cystine are formed from each other and can form pyruvate through B-mercaptopyruvate (Cantarow and Schepartz 1960).

The Effect of Malathion on the Metabolism of Labelled Compounds:

Malathion intoxication caused an increased metabolic activity in the poisoned roaches (also discussed in previous sections). This has resulted in greater production of $C^{14}O_2$, depleting both the exogenous carbohydrate, protein, and amino acid reserves. The increased metabolic activity of poisoned roaches, as compared to the controls, is evidenced by higher production of $C^{14}O_2$: about 73 per cent in glucose (U) C^{14} experiments, about 84 per cent in glutamate (U) C^{14} experiments and about 46 per cent in glycine (U) C^{14} experiments (Tables V, VIII, XI). These observations indicate enhanced glycolytic and Krebs' cycle activity in the poisoned roaches.

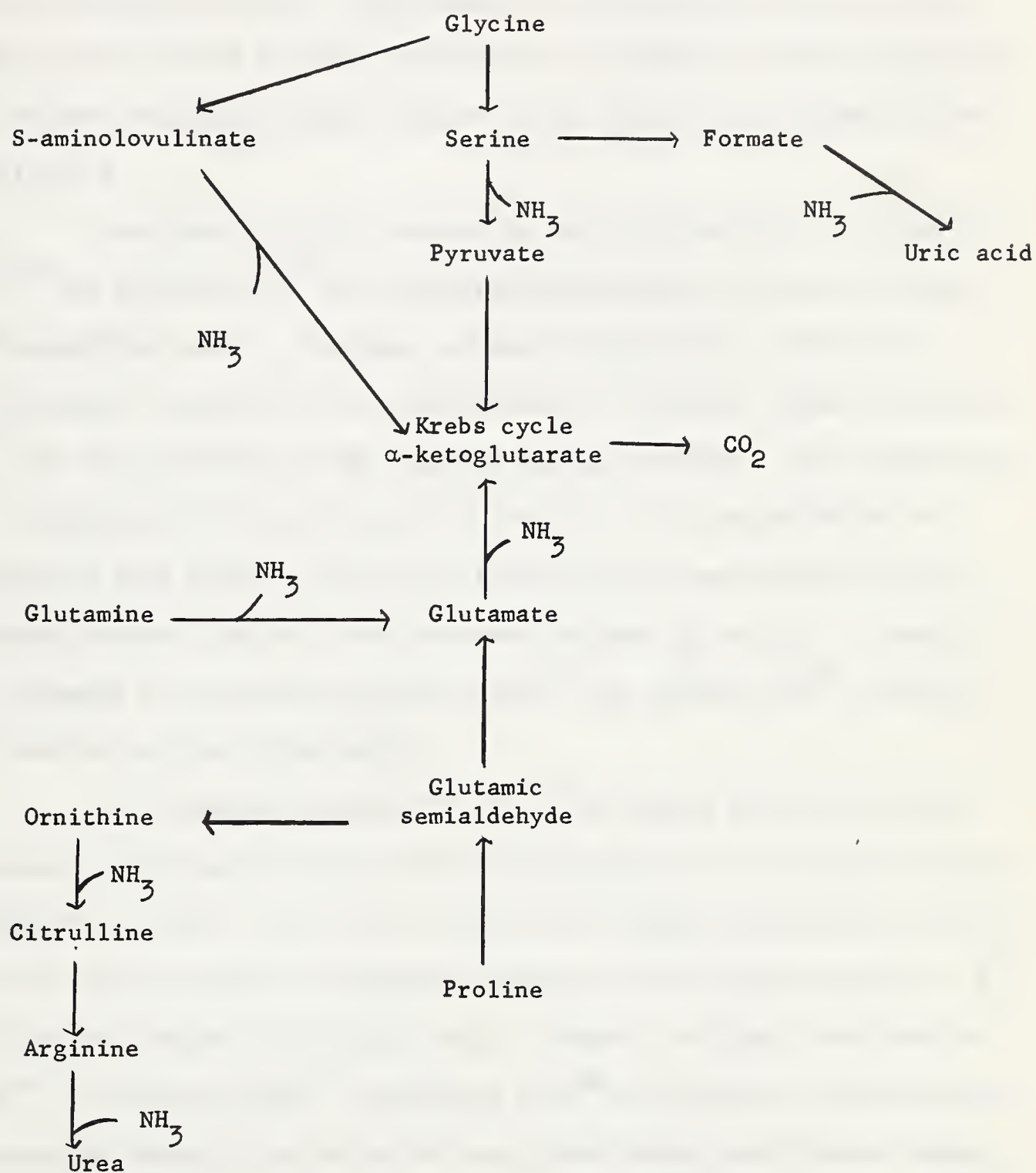
The susceptible strain roaches, treated with malathion were found to have undergone reduction of carbohydrate reserves. They consumed about 59 per cent more of glucose (U) C^{14} than the controls (Table V). Trehalose was also depleted in the blood (48 per cent) and body (80 per cent) of the poisoned roaches. About 68 per cent reduction was observed in glycogen of intoxicated roaches (Table VI). Similarly, malathion toxicity resulted in over 60 per cent less incorporation of labelled carbon of glutamate (U) C^{14} into sugars (Table IX) and about 24 per cent less into glycogen of the poisoned roaches as compared to the controls. These observations suggest that most of glutamate that entered the Krebs' cycle as α -ketoglutarate, was oxidised to water and $C^{14}O_2$ rather than being used for carbohydrate synthesis.

There was no significance difference in the incorporation of C^{14} of glycine (U) C^{14} into body sugar or glycogen in the poisoned and unpoisoned roaches.

Malathion toxicity decreased the incorporation of labelled carbon of glucose (U) C^{14} into protein and amino acids (Table V). Free glutamin-asparagine, glycine, proline, and glutamate showed lower incorporation of C^{14} in the poisoned roaches than the controls (Table VII). This indicates greater utilization of free amino acids in poisoned roaches. Moreover, there may also be less availability of substrate for amino acids synthesis, as most of the glucose would be metabolised to $C^{14}O_2$ and water, particularly in impaired Krebs' cycle (O'Brien 1957).

Incorporation of C^{14} of glutamate (U) C^{14} and glycine (U) C^{14} into protein was not affected by malathion. However, incorporation of labelled carbon of glutamate (U) C^{14} into glycine, proline and arginine (Table X)

Figure 8. Hypothetical pathways of glycine, proline, glutamate and glutamine catabolism in malathion intoxicated B. germanica.



and that of glycine (U) C^{14} into glutamate (Table XIII) was reduced by malathion. Besides, the intoxicated insects utilized about 19 and 22 per cent more of labelled glutamate and glycine respectively, than the controls (Table X and XI). The effect of insecticide on the metabolism of these amino acids has been discussed in the previous section (page 55). The present observations give support to the hypothetical pathways shown in Figure 8 .

There was a slight increase in the incorporation of glutamate (U) C^{14} and glycine (U) C^{14} into glutamine-asparagine of poisoned roaches of susceptible strain. Corrigan and Kearns (1963) also observed an insignificant increase in the incorporation of labelled carbon of proline C^{14} into the glutamine of DDT poisoned American roaches. This observation is consistent with the effect of malathion on the concentration of endogenous free glutamine which was found to have been reduced in the poisoned roaches (Table II and discussed on pages 55 and 56). Probably the presence of unutilized glutamate (U) C^{14} and glycine (U) C^{14} affected the results in these experiments.

An increased incorporation of C^{14} of almost all the injected compounds into organic acid fraction was observed in the poisoned roaches. Probably the loss of some of the organic acid during experimental procedure did not show any effect of malathion toxicity on the incorporation of C^{14} of labelled glucose into organic acid. However, increased incorporation of C^{14} of glutamate (U) C^{14} and glycine (U) C^{14} into organic acid fractions indicate an enhanced conversion of these amino acids into certain organic acids, in malathion poisoned roaches. Glycine (U) C^{14} , for instance, could be deaminated to glyoxylate, resulting in greater ammonia liberation in

poisoned insects than in controls. (Discussed on page 35).

The Effect of Malathion on the Metabolism of Labelled Compounds
in the Resistant Strain, Compared with the Susceptible Strain:

The malathion treated roaches of the resistant strain, though subjected to the same concentration of insecticide as the susceptible strain, were observed to metabolize labelled glucose glutamate and glycine in a similar manner as the untreated controls (Tables V and XIII). There was, however, a slight increase in the output of $C^{14}O_2$ in the resistant strains than controls. In the glucose $(U)C^{14}$ experiments, almost all of the substrate for the increased metabolic activity was derived from glucose (27 per cent), trehalose (15 per cent) and blood glutamate (30 per cent). The poisoned roaches of the resistant strain, injected with glutamate $(U)C^{14}$ produced about 12 per cent more of $C^{14}O_2$, depleting free amino acids of the blood by about 27 per cent and sugar by about 30 per cent as compared to the controls. A similar situation was observed with glycine $(U)C^{14}$ experiments.

Thus, susceptible and resistant insects, treated with the same concentration of malathion, derive substrate for increased metabolic activity, from one or more sources. While the resistant strain undergo depletion mostly in carbohydrate reserves, the susceptible strain utilizes fairly large amounts of carbohydrates and free amino acids, and to some extent, protein. This shows a direct correlation between the depletion of some amino acids and the degree of intoxication of roaches by malathion.

Almost complete inhibition of the activity of brain cholinesterase was observed in malathion poisoned roaches of the susceptible strain, while the roaches of the resistant strain had only about 30 per cent inhibition of the enzymic activity (Figure 2 of the appendix). Mengle and Casida (1960) demonstrated the presence of a large concentration of a "factor" in a resistant strain of houseflies which protected the inhibition of cholinesterase activity. A similar protection mechanism against malathion intoxication is probably present in the resistant strains of German roaches. This "factor" for resistance is also responsible for protecting the roaches of the resistant strains against intoxication, to the extent that amino acids are not utilized for greater energy production.

4. Conclusion:

Labelled carbon of injected glucose $(U)C^{14}$, glutamate $(U)C^{14}$ and glycine $(U)C^{14}$ was incorporated mainly into $C^{14}O_2$, organic acids and sugar phosphates, amino acids, protein, carbohydrate and lipids. These observations suggest mammalian-like breakdown of the injected compounds through the Embden-Meyerhof sequence of glycolysis and the Krebs' TCA cycle.

The present studies indicate the presence of an efficient mechanism of transamination and carbohydrate-amino acid interconversion in German roaches. The labelled carbon of glutamate ~~and~~ $(U)C^{14}$ were incorporated into the following amino acids: glutamate, aspartate, glutamine-asparagine, proline, alanine, glycine and serine. Arginine, however, incorporated labelled carbon only from glutamate $(U)C^{14}$. All the above mentioned amino acids (except arginine) along with cystine/cysteine as well as the tripeptide glutathione incorporated the labelled carbon of glycine $(U)C^{14}$.

Malathion intoxication increased the catabolism of the injected compounds resulting in greater production of $C^{14}O_2$ and depletion of carbohydrate, amino acids and protein reserves in the poisoned roaches.

The poisoned roaches of the resistant strain catabolised the injected compounds almost in a similar manner as the unpoisoned roaches of the susceptible strain.

It is concluded that the depletion of amino acids and carbohydrates in the poisoned roaches is a consequence of intoxication. These compounds are utilized via the Krebs' cycle for greater energy production.

VI GENERAL DISCUSSION AND CONCLUSION

The mode of action of malathion on the susceptible and resistant strains of B. germanica was investigated. An attempt was made to relate the gross symptoms of intoxication with some of the more specific physiological and biochemical changes in the insect.

Malathion intoxicated roaches of the susceptible strain showed hyperactivity and incoordination resulting in knockdown of the insects followed by tremors, paralysis and death. Associated with the intoxication were the phenomena of increased regurgitation, defecation and water loss. The inefficiency of the water regulation mechanism in poisoned roaches was manifested by the depletion of the blood. There was a direct positive correlation between water loss and malathion intoxication. After six hours of poisoning, the paralysed group lost about 70 per cent of the blood volume, while the unparalysed group lost only about 36 per cent. The increased regurgitation and defecation accounted for about 30 per cent of the loss in wet weight of the poisoned roaches. However, most of the wet weight was lost in the form of water as a result of increased transpiration through the spiracles and intersegmental membranes, which were exposed due to the collapse of the muscular system.

Malathion intoxication induced hyperactivity with the resulting increase in oxygen uptake. An increased metabolic activity of intoxicated roaches was also shown by increased catabolism of administered glucose (U) C^{14} , resulting in the production of about 73 per cent more of $C^{14}O_2$ than the controls. The substrate for the increased respiration was derived from

reserve carbohydrate, protein and amino acids, causing the intoxicated roaches to lose about 7 per cent of their dry weight. The poisoned roaches used more of glucose $(U)C^{14}$ than the controls and incorporated less of labelled carbon into trehalose and glycogen.

However, the poisoned roaches could not be expected to depend only upon carbohydrates reserves for greater energy production, particularly if the insecticide inhibits glycolytic breakdown of glucose. Even if considerable respirable carbohydrate substrate were available, the slow synthesis of pyruvate from glucose could force a shift in the substrate for the Krebs' cycle, from carbohydrates to free amino acids which are present in very high concentrations. The experiments indicate that the depletion of amino acids was caused mainly by their utilization via the Krebs' TCA cycle. This view is supported from the observation that poisoned roaches of susceptible strain, administered with glutamate $(U)C^{14}$ lost about 84 per cent more $C^{14}O_2$ than the controls. Similarly those injected with glycine $(U)C^{14}$ released about 46 per cent more of $C^{14}O_2$ than the unpoisoned controls. This greater production of $C^{14}O_2$ was accompanied by greater utilization of injected glutamate $(U)C^{14}$ and glycine $(U)C^{14}$. Further evidences of utilization of free amino acids by the Krebs' cycle are afforded by the incorporation of labelled carbon from each of glucose $(U)C^{14}$, glutamate $(U)C^{14}$ and glycine $(U)C^{14}$ into carbohydrates, amino acids and protein of poisoned and unpoisoned roaches of both the resistant and susceptible strains.

The blood of B. germanica was found to contain 18 free amino acids making up a total concentration of about 247 mg per 100 ml of blood. Glycine, glutamine and proline were the amino acids present in highest

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concentrations, contributing about half of the size of the amino acid pool of the blood. Malathion toxicity did not result in any change in the concentration of 14 of the 18 free amino acids of the blood. However, five and a half hours of malathion intoxication was found to have reduced the concentrations of free proline; glycine, glutamate and glutamine by, at least, 70 per cent. The depletion had a direct positive correlation with the poisoning; the most intoxicated susceptible paralysed group lost the greatest amount of amino acids, followed by susceptible unparalysed group and the least intoxicated resistant group. There was also a selective correlation between the onset of toxic symptoms and the depletion of some amino acids. Even the slightest intoxication induced depletion of glycine at a rate faster than that of any other amino acid. However, as the poisoning increased, other amino acids, particularly proline, reduced at a faster rate than glycine. Glutamine was not reduced in the malathion treated resistant roaches. It appears that malathion does not affect glutamine concentration until the increased poisoning reduced concentrations of some other amino acids to a low level.

Malathion-treated roaches of the susceptible strain had incorporated less labelled carbon from glucose $(U)C^{14}$ into body protein and free glutamine-asparagine, glutamate, glycine and proline, than the controls. The utilization of glucose $(U)C^{14}$ for greater energy production, resulting in liberation of more $C^{14}O_2$ in poisoned roaches is likely to make less substrate available for amino acid synthesis. A similar mechanism explains the lowered incorporation of C^{14} of glutamate $(U)C^{14}$ into carbohydrates of the poisoned roaches.

In the normal roaches, labelled carbon of glutamate $(U)C^{14}$ was incorporated into aspartate, glutamine-asparagine, proline, alanine, serine, glycine and arginine. In the poisoned susceptible strain, proline, glycine and arginine incorporated much less C^{14} than in the controls. This decreased incorporation of C^{14} into proline, even though a fairly large amount of glutamate $(U)C^{14}$ was present in the poisoned roaches, suggests the possibility that malathion intoxication facilitates the conversion of proline into glutamate. The effect of malathion on proline is also evidenced by the recovery of this amino acid in the poisoned resistant roaches after initial depletion.

The intoxicated roaches showed less incorporation of C^{14} of glycine $(U)C^{14}$ into glutamate, whereas there was no difference in the incorporation of labelled carbon into free glutamine-asparagine, aspartate, alanine, serine, cystine/cysteine between the treated and untreated roaches of susceptible strain.

The incorporation of labelled carbon of glucose $(U)C^{14}$, glutamate $(U)C^{14}$ and glycine $(U)C^{14}$ into carbohydrate, amino acids, protein and carbon dioxide suggests the existence of mammalian-like pathways of carbohydrate and amino acid metabolism. Most of the glycine could thus be converted to pyruvate for final utilization via the Krebs' cycle. Proline, glutamine and glutamate would be expected to supply a substrate for the Krebs' cycle.

Another major pathway of the depletion of amino acids, particularly proline, is expected to be urea formation via the ornithine cycle, as is evidenced by increased blood urea of the poisoned roaches. Participation of glycine in the synthesis of uric acid may have caused the observed

increase of total nitrogen in the blood of the intoxicated roaches.

The depletion of free glutamine suggested complete break-down of this system of ammonia disposal in poisoned roaches of the susceptible strain. The increase of total nitrogen and non-protein nitrogen in the poisoned roaches indicate the accumulation of nitrogenous metabolites.

Very little effect of malathion intoxication was observed on the free amino acids of the blood of the resistant strain roaches. The slight depletion of glycine, proline and glutamate and the subsequent recovery of proline and glutamate may be compared with the in vivo inhibition of cholinesterase due to mild poisoning and its subsequent recovery in the poisoned roaches. Experiments with glucose (U) C^{14} , glutamate (U) C^{14} and glycine (U) C^{14} do not show any significant difference between the poisoned roaches of resistant strain and the unpoisoned controls, in the incorporation of labelled carbon of any of the above mentioned compounds, into carbohydrate, protein, amino acids and carbon dioxide. These observations suggest the presence of some mechanism in the roaches of resistant strain which protects both the inhibition of cholinesterase activity and the depletion of amino acids, from malathion toxicity.

The depletion of free amino acids and protein in the poisoned roaches is likely to affect the efficiency of blood in maintaining the pH and the osmotic pressure. The accumulation of ammonia and nitrogenous metabolites would affect biochemical reactions. It is, however, hard to say at this stage as to how far the various physiological reactions would be affected. A detailed study of the effect of malathion on the physiology of excretion would help the evaluation of the present findings.

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Introduction

The purpose of this study is to investigate the effects of various factors on the growth and development of the human body. The study is designed to provide a comprehensive overview of the factors that influence human growth and development, including genetic, environmental, and nutritional factors.

The study is divided into two main sections. The first section, titled "Genetic Factors," discusses the role of genetics in human growth and development. The second section, titled "Environmental Factors," discusses the role of environmental factors in human growth and development.

The first section, titled "Genetic Factors," discusses the role of genetics in human growth and development. It covers the following topics: the role of chromosomes, the role of genes, and the role of the environment in the expression of genes.

The second section, titled "Environmental Factors," discusses the role of environmental factors in human growth and development. It covers the following topics: the role of nutrition, the role of exercise, and the role of stress in human growth and development.

The study is designed to provide a comprehensive overview of the factors that influence human growth and development. It is intended for use by students, researchers, and anyone interested in the field of human growth and development.

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APPENDIX

<u>Materials</u>	<u>Sources</u>
Acetic acid glacial.	Fisher Scientific Co. Ltd., Canada.
Acetyl choline bromide. Highest Purity.	Nutritional Biochemical Corporation. Cleveland, Ohio.
Amino acids - L.	Same as above.
n-butanol.	Fisher Scientific Co. Ltd., Canada.
Butyl-acetate.	Same as above.
Glucose (U) C^{14} .	Atomic Energy of Canada Ltd., Ottawa.
L-Glutamic Acid (U) C^{14} .	Same as above.
Glycine (U) C^{14} .	Same as above.
2 - 6 - Lutidine.	Fisher Scientific Co. Ltd., Canada.
Malathion - 95% technical.	American Cyanamid Company, New York.
Methyl cellosolve.	Fisher Scientific Co. Ltd., Canada.
Ninhydrin.	Nutritional Biochemicals Corporation.
Phenol - liquified.	Fisher Scientific Co. Ltd., Canada.
Iso-propyl alcohol.	Same as above.

Resins:

Analytical grade anion exchange Dowex 1 x 10 chloride form. 200- 400 mesh, chromatography grade.	California Corporation for Biochemical Research, Los Angeles.
Nalcite HCR-'H' form. 15 - 30 mesh, 8 - 12% cross linkage.	Aluminate Corporation, U.S.A.
2-4-6-Trimethylpyridine.	Fisher Scientific Co. Ltd., Canada.

SELECTION OF B. GERMANICA FOR RESISTANCE TO MALATHION

A strain of German roaches that had acquired about 9 fold resistance to malathion was obtained from Dr. James Mcd. Grayson, Virginia Agricultural Experimental Station, Blacksburg, Virginia. The resistance was further increased and maintained by the method of Grayson (1960). Third and fourth stage nymphs of every generation were released on filter paper treated with 10 ml of malathion in acetone (see page 7). An exposure time of one hour on the treated surface resulted in about 50 per cent mortality. However, after five generations, nymphs were exposed for 70 to 80 minutes to obtain the same mortality. In order to avoid variation in biochemical investigations, an attempt was made to maintain the degree of resistance acquired by the roaches. This was done by appying selection pressure to every alternate generation, after five successive generations were exposed to malathion pressure. The roaches were reared in the usual way (page 7).

Test for Degree of Resistance:

The degree of resistance was determined by comparing the 24 hours LC 50 of malathion for 15-20 days old adult males of the susceptible and resistant strains. In order to get mortalities between 10 and 80 per cent, the susceptible strain was treated with 0.1, 0.2, 0.4, 0.6 and 0.8 per cent malathion in acetone. The resistant strain was treated with 4, 6, 8 and 10 per cent malathion. The roaches were released on treated filter paper according to the method described on page 7. The treated roaches were kept at 30°C and 50-60 per cent relative humidity and were provided with food and water. Twenty four hours later, the mortalities

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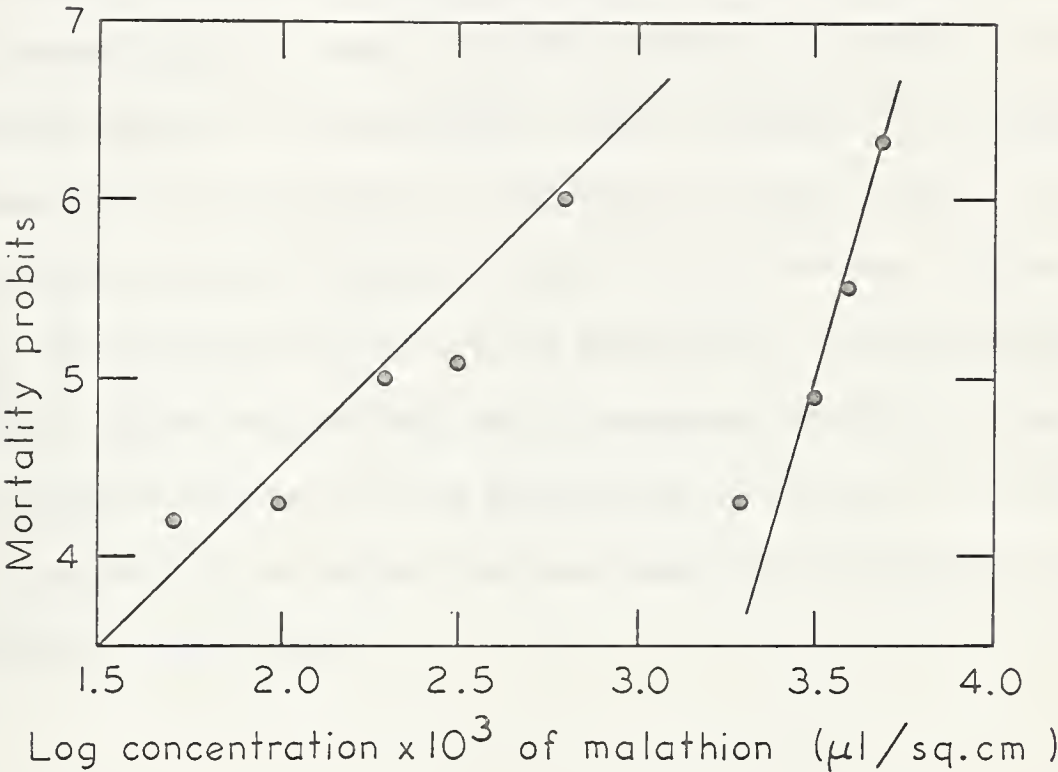
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Table I-A
LC50 (24 hours) of malathion to adult male B. germanica
of susceptible and resistant strains.

Strains	Heterogeneity	Regression equation	LC50	Fiducial limits	Relative resistance
Susceptible	χ^2 2.04 (4)	$Y = 0.8 - 1.87x$	0.18	0.187 0.173	1
Resistance	χ^2 2.76 (3)	$Y = 18.7 - 6.8x$	3.09	3.091 3.089	17.2

Y Probit mortality.
x Log of concentration $\times 10^3$.
L The data were homogeneous at $P = 0.05$.
LC50 Concentration in microlitres of malathion per square centimetre of filter paper, calculated to give 50 per cent mortality.

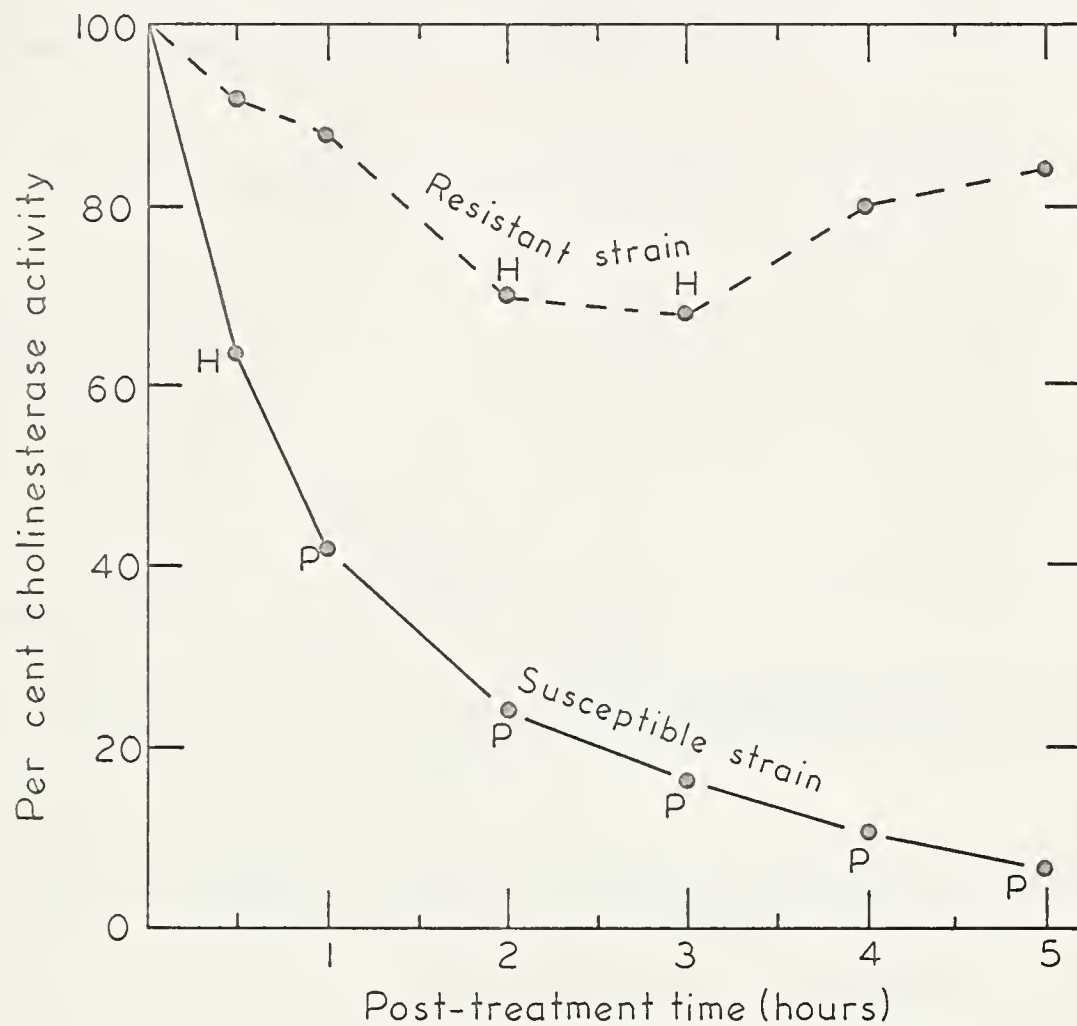
Figure 1. Mortality of susceptible and resistant strains of *B. germanica* on malathion treatment.



were counted. The data were statistically analysed as described by Busvine (1957).

Results and Discussion: The results are presented in Figure I and Table I. The 24 hour LC50 or the concentration of malathion expected to give fifty percent mortality of the susceptible strain was found to be 0.18 microlitres of malathion per square centimetre of filter paper surface, while that of the resistant strain was 3.09 microlitres. Thus a strain of German roaches was obtained which was about 17 fold resistant to malathion. The flat regression line for the susceptible strain indicates the heterogeneity of the population in the ability to withstand the fatal effects of malathion. On the other hand, the regression line for the resistant strain was steep, showing the homogenous nature of the population in tolerance towards malathion. These observations are in agreement with Hoskins and Gordon (1956) who have pointed out the possibility of the use of the slope of regression line in predicting the development of resistance by insects to different insecticides.

Figure 2. The effect of malathion on the *in vivo* activity of brain cholinesterase of resistant and susceptible strains of *B. germanica*

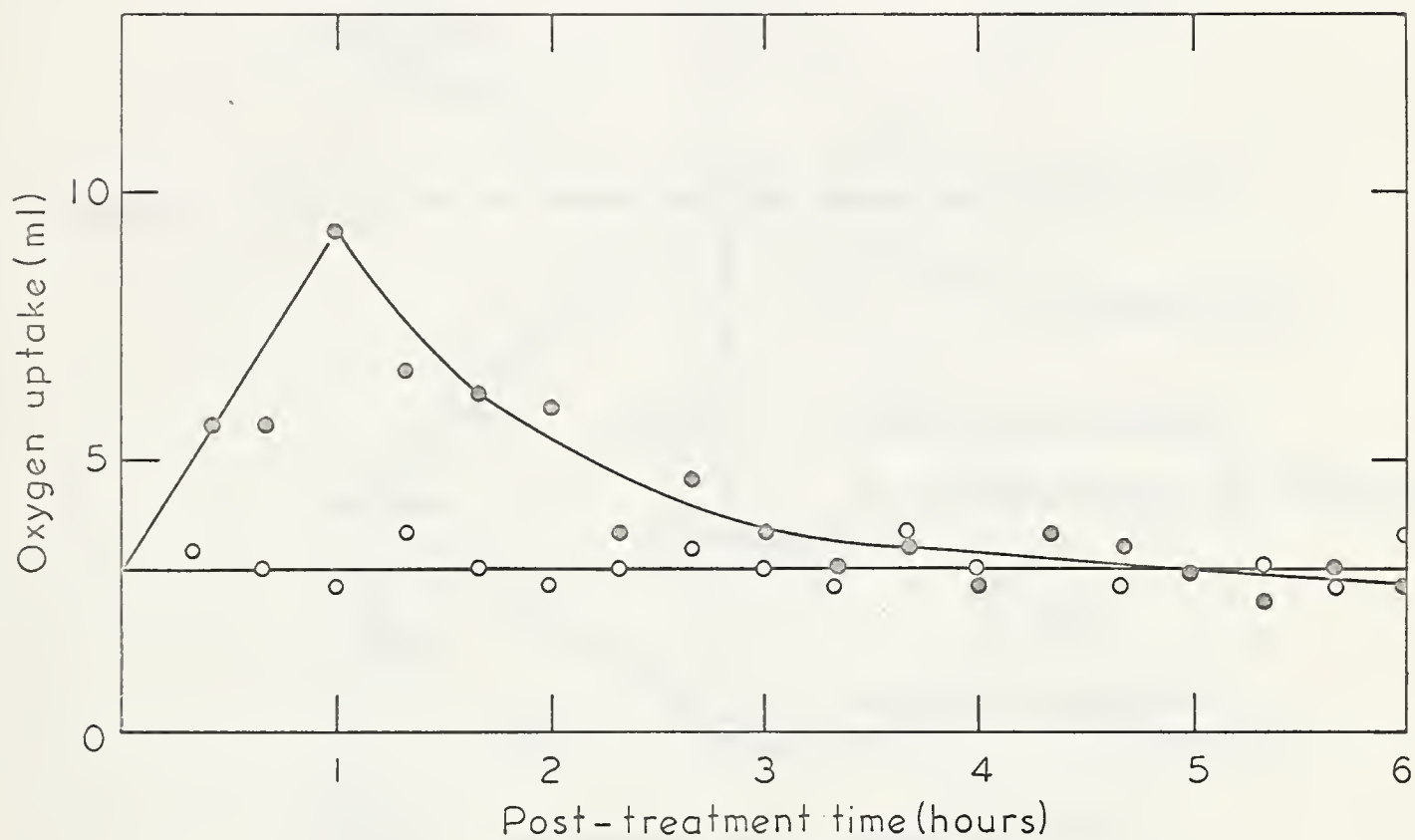


P Paralysed

H Hyperactive

* Dose, (0.46 μ l/ sq. cm.), lethal to susceptible strain in seven hours

Figure 3. Effect of malathion* on oxygen uptake by *B. germanica*



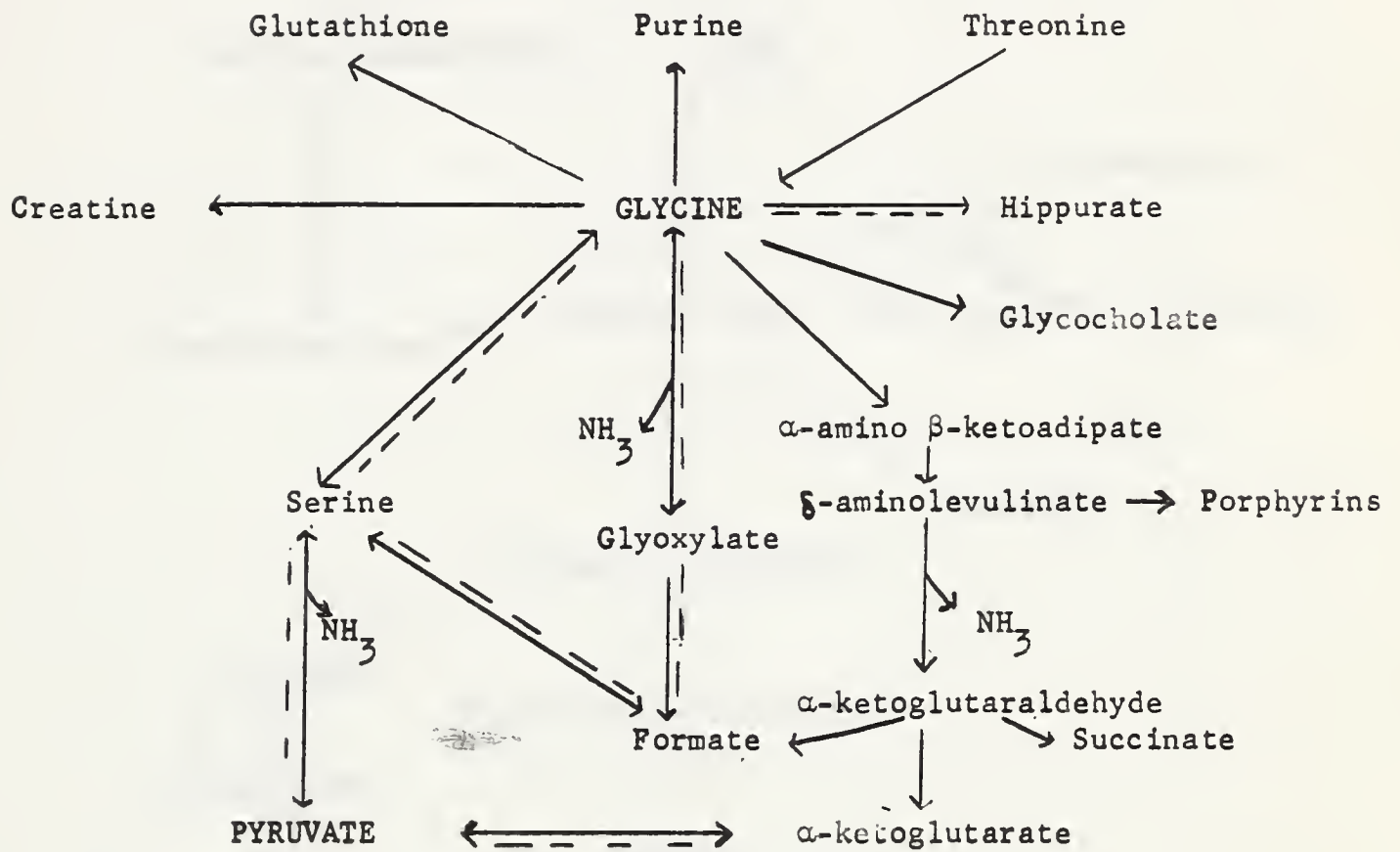
* Dose lethal in seven hours ($0.46 \mu\text{l/sq. cm.}$)

● — ● Malathion treated roaches

○ — ○ Control roaches

Figure 4

Major Pathways of Glycine Metabolism
in Mammals and Insects.



Legend:

- In mammals (Cantarow and Schepartz 1958)
- In insects (reported so far - see page 35).

Figure 1

Relationships among variables in the conceptual model

Legend: $\rightarrow$ Hypothesis



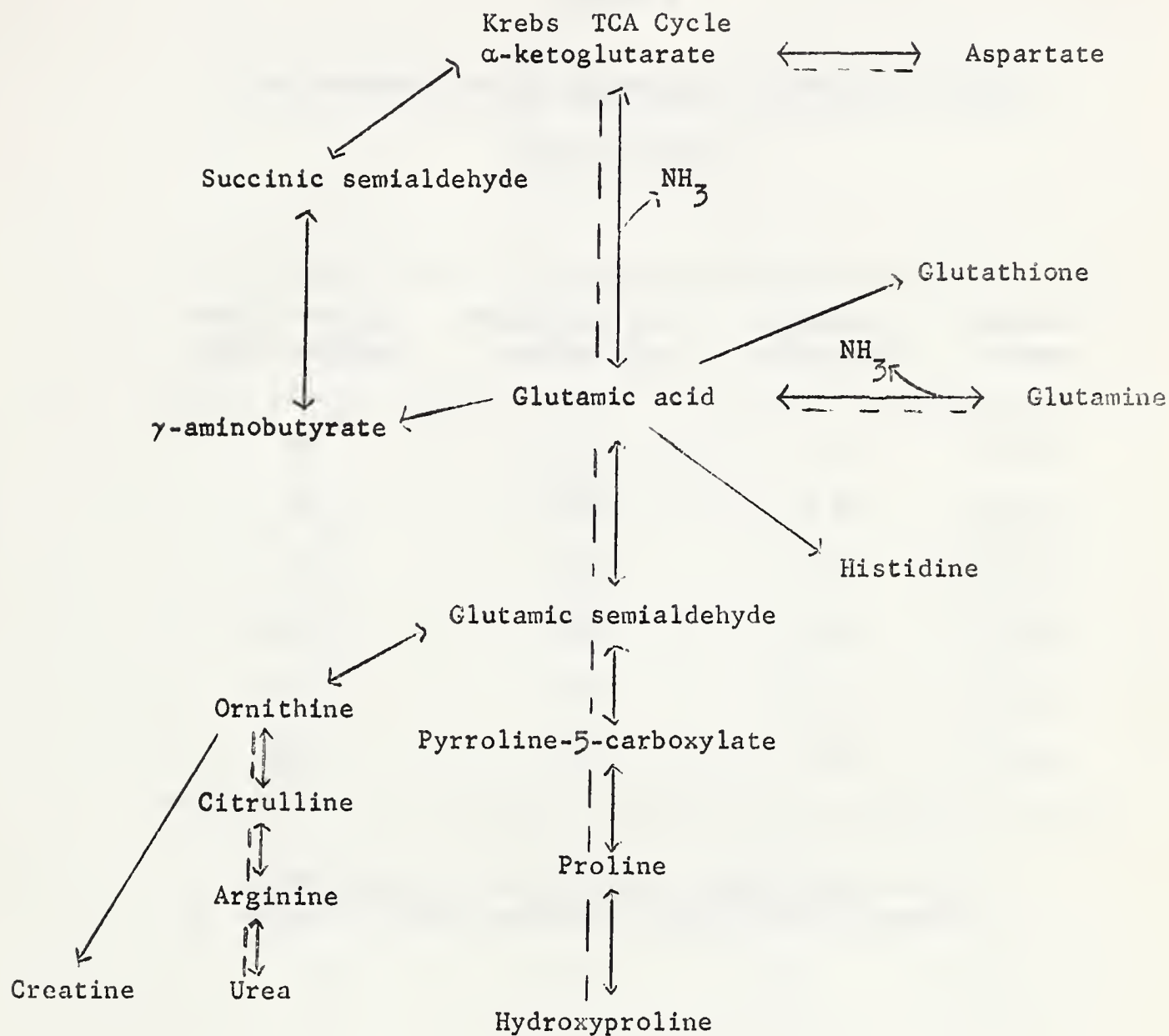
Figure 1. Relationships among variables in the conceptual model

Legend: $\rightarrow$ Hypothesis

Figure 5

Major Pathways of Proline and Glutamate

Metabolism in Mammals and Insects.



Legend:

————— In mammals (White et al. 1959).

- - - - - In insects (reported so far) see page 36 .

Table I

The knockdown of B. germanica (susceptible strain)
by malathion.¹

Post treatment time (minutes)	Mean ² per cent knocked down	Standard deviation	Standard error
30	8	5.4	3.75
60	25	3.6	2.60
90	40	3.3	2.30
180	75	7.6	5.28
270	90	5.7	3.96
360	97	1.4	0.98

1 Dose lethal in seven hours (0.46 μ l/sq. cm.)

2 Mean of three replicates, each with 30 insects.

TABLE I
Summary of the results of the experiments on the effect of the concentration of the solution on the rate of polymerization of styrene in benzene at 50°C.

Conc. of styrene (mole/l.)	Conc. of benzene (mole/l.)	Conc. of K ₂ S ₂ O ₈ (mole/l.)	Rate of polymerization (mole/l. hr.)
0.01	0.01	0.01	0.01
0.02	0.02	0.02	0.02
0.03	0.03	0.03	0.03
0.04	0.04	0.04	0.04
0.05	0.05	0.05	0.05
0.06	0.06	0.06	0.06
0.07	0.07	0.07	0.07
0.08	0.08	0.08	0.08
0.09	0.09	0.09	0.09
0.10	0.10	0.10	0.10

The rate of polymerization was measured by the method of measuring the amount of polymer formed in a given time.

Table II

The reduction in the volume of hemolymph of
malathion treated¹ B. germanica .

Post treatment time (minutes)	Mean ² % reduction	Standard deviation	Standard error
<u>Paralysed group</u>			
60	24.7	1.10	± 0.76
120	45.2	1.4	± 0.98
240	65.9	4.0	± 2.78
360	80.0	4.6	± 3.19
<u>Unparalysed group</u>			
60	9.7	2.08	± 1.44
120	15.0	2.00	± 1.39
240	22.3	2.60	± 1.81
360	45.0	4.36	± 3.03
<u>Whole population</u>			
60	12.3	2.60	± 1.80
120	27.0	2.60	± 1.80
240	58.7	6.10	± 4.20
360	68.3	6.04	± 4.19
<u>Control</u>			
120	1.5	0.86	± 0.60
240	5.3	2.6	± 1.8
360	8.7	1.6	± 1.1

1 Dose lethal in seven hours (0.46 µl/sq. cm.).

2 Mean of three replicates.

Table III

The reduction in wet weight of malathion¹
treated B. germanica.

Post treatment time (minutes)	Mean ² % reduction	Standard deviation	Standard error
<u>Treated group</u>			
60	6.51	0.36	0.25
120	9.68	0.88	0.6
180	13.00	2.00	1.39
240	15.00	2.14	1.49
300	18.17	1.48	1.00
360	21.50	1.76	1.22
<u>Control</u>			
60	1.09	0.13	0.09
120	1.50	0.46	0.32
180	1.80	0.28	0.19
240	2.50	0.12	0.09
300	3.10	0.28	0.19
360	3.80	0.46	0.32

- 1 Dose lethal in seven hours (0.46 μ l/sq. cm.).
2 Mean of three replicates.

TABLE

Summary of the results of the analysis of variance for the

analysis of variance for the

Analysis of Variance for the			
Source of Variation	Sum of Squares	D.F.	Mean Square
Between Groups			
1	18.5	1	18.5
2	19.5	1	19.5
3	18.5	1	18.5
4	19.5	1	19.5
5	18.5	1	18.5
6	19.5	1	19.5
Within Groups			
1	18.5	1	18.5
2	19.5	1	19.5
3	18.5	1	18.5
4	19.5	1	19.5
5	18.5	1	18.5
6	19.5	1	19.5

Source: *Journal of the American Statistical Association*, 1954, 49, 1000-1001.

Table IV

The effect of ligaturing on wet weight loss
in malathion¹ treated B. germanica.

Post treatment time (minutes)	Mean ² % reduction	Standard deviation	Standard error
<u>Treated ligatured</u>			
60	3.65	0.45	0.31
120	5.84	0.48	0.33
180	8.83	0.80	0.56
240	11.42	0.80	0.56
300	13.16	0.34	0.24
260	15.76	0.28	0.19
<u>Control ligatured</u>			
60	1.00	0.199	0.138
120	1.54	0.11	0.760
180	1.85	0.17	0.118
240	2.23	0.09	0.063
300	2.74	0.103	0.072
360	3.35	0.48	0.33

1 Dose lethal in seven hours (0.46 μ l/sq. cm.).

2 Mean of three replicates.

TABLE I

Summary of the results of the analysis of variance for the effect of the different factors on the yield of the different components of the plant.

Factor	Level	Yield of the plant (g)	Yield of the components (g)
Irrigation (mm)			
0	0	10.0	0.0
10	10	15.0	5.0
20	20	20.0	10.0
30	30	25.0	15.0
40	40	30.0	20.0
50	50	35.0	25.0
60	60	40.0	30.0
70	70	45.0	35.0
80	80	50.0	40.0
90	90	55.0	45.0
100	100	60.0	50.0
Fertilization (g)			
0	0	10.0	0.0
10	10	15.0	5.0
20	20	20.0	10.0
30	30	25.0	15.0
40	40	30.0	20.0
50	50	35.0	25.0
60	60	40.0	30.0
70	70	45.0	35.0
80	80	50.0	40.0
90	90	55.0	45.0
100	100	60.0	50.0

Table V

Rf Values of Amino Acids in Different Solvent Systems.

Amino acids	Solvent systems				
	1	2	3	4	5
Lysine	0.81	0.92	-	0.07	0.78
Histidine	0.75	0.1	-	0.23	0.72
Arginine	0.75	0.1	-	0.07	0.78
Glycine	0.51	0.16	0.30	0.20	0.40
Alanine	0.45	0.26	0.45	0.24	0.60
Valine	0.34	0.47	-	0.34	0.73
Serine	0.37	0.16	0.24	0.21	0.35
Leucine/isoleucine	0.34	0.62	-	0.43	0.73
Threonine	0.33	0.20	0.40	0.30	0.53
Glutamine	0.27	0.14	0.13	0.12	0.56
Asparagine	0.24	0.12	0.12	0.12	0.40
Glutamate	0.27	0.21	0.15	0.10	0.30
Aspartate	0.20	0.15	0.10	0.10	0.18
Proline	0.26	0.31	-	0.27	-
Tyrosine	0.23	0.4	-	0.52	0.59
Methionine	0.23	0.56	-	0.45	0.73
Phenylalanine	-	-	-	0.45	0.73
Cystine	0.18	0.06	-	-	-
β -alanine	0.46	0.25	-	0.15	0.64
Glutathione	0.08	-	-	-	-

Solvent systems

- 1 High voltage electrophoresis (Richmond and Hartley 1959)
- 2 Butanol - butyl acetate (Richmond and Hartley 1959)
- 3 Phenol pH12 (McFarren 1951)
- 4 Lutidine - Collidine (Block 1958)
- 5 Phenol water saturated (Block 1958)



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